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MASSACHUSETTS UNIV AMHERST DEPT OF MICROBIOLOGY
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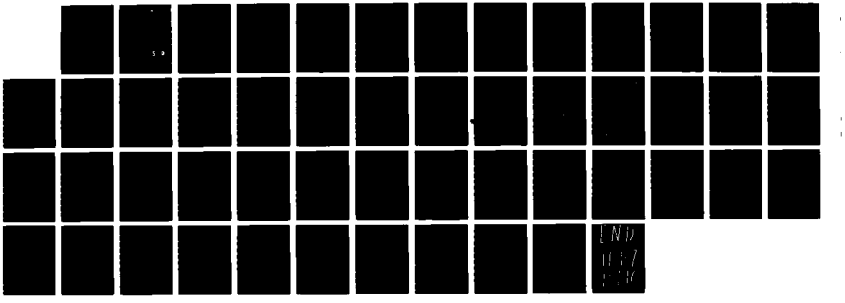

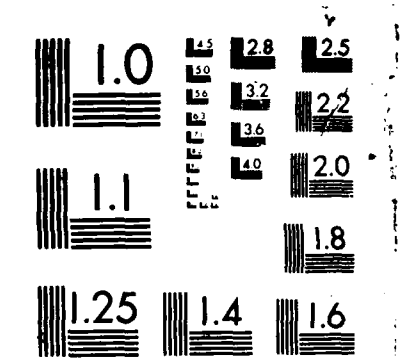

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CHEMOTHERAPY AND BIOCHEMISTRY OF LEISHMANIA

ANNUAL REPORT

LINDA L. NOLAN, Ph.D.

DECEMBER 1984

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1198

University of Massachusetts
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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A185457	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Chemotherapy and Biochemistry of Leishmania		5. TYPE OF REPORT & PERIOD COVERED Annual Report Jan.1,1984-Dec.31,1984
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Linda L. Nolan, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD17-8i-C-1198
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology University of Massachusetts Amherst, MA 01003		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102-A-3M161102BS10.AF.080
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command; Fort Detrick, Frederick, MD 21701-5012		12. REPORT DATE December 1984
		13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Leishmania/mode of action purine anologs

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to synthesize purines de novo and consequently are obligate salvagers of preformed purines.

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Summary

On May 17, 1984 a workshop on antileishmanial drug development was set up at WRAIR for the purpose of establishing a major intermural, multidisciplinary drug development research effort. This antileishmanial research program is being directed through the Division of Experimental Therapeutics, and includes three laboratories at WRAIR and three U.S. university laboratories. The role of this laboratory in the program is to test compounds in vivo using different Leishmania spp. and to determine the molecular mode of action of promising compounds. Compounds provided by WRAIR were tested singly, and for synergy, in combination.

The most promising compounds to date sent by WRAIR appear to be the following:

BK63005 3-B-D-Ribofurano-sylpyrazolo-[4,3-d] pyrimidin-7-thione
BK74731 oxoformycin B
BK63863 Thiopurinol riboside
BK71338 oxoformycin A
BK86124 Allopurinol riboside
9-Deazainosine

The following compounds sent by Dr. Peter K. Chiang (Department of Biochemistry, WRAIR) were found to be very inhibitory to promastigotes of Leishmania mexicana amazonensis WR227 : 4'-thioadenosine, deoxyaristeromycin and 5-deoxy-5 (isobutylthio)-3-deazaadenosine (deaza-SIBA).

Sinefungin, a naturally occurring antifungal nucleoside antibiotic containing an ornithine residue, obtained from Dr. M. Robert-Gero (ICSN-CNRS, Gif Sur Yvette, France) was also found to be very inhibitory.

A DNA-dependent RNA polymerase has been isolated and characterized from Leishmania mexicana WR #227. The initial stages of purification utilized high ionic strength extraction and protamine sulfate treatment. The enzyme was further purified by differential elution on Heparin-Sepharose, DEAE-Sephadex, and Carboxymethyl-Sephadex chromatography. Analysis of the chromatographically purified RNA polymerase on nondenaturing gels revealed two electrophoretic forms. The enzyme isolated has characteristics of true DNA-dependent RNA polymerase since it requires DNA and all four nucleoside triphosphates for the synthesis of RNAase-sensitive products. Analysis of ammonium sulfate and metal ion optima, as well as relative activities of the enzyme with Mn^{2+} versus Mg^{2+} are similar to those reported for other RNA polymerase III in eukaryotes.

Formycin A triphosphate was found to be a competitive substrate for this enzyme, and cordycepin triphosphate was found to be inhibitory, although the mode of inhibition was not determined.

We have partially purified a DNA polymerase from L. mexicana WR #227 which is N-ethylmaleimide sensitive, aphidicolin resistant and showed different sensitivities to 2-acrylaminopurine deoxyribonucleoside-5'-triphosphate than mammalian DNA polymerase α. The purification scheme resulted in removal of over 99% of protein with over a 282 fold increase in specific activity.

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RESUME OF PROGRESS

Culture Methods:

The organisms used in this project have been obtained from the Walter Reed Army Institute of Research through the courtesy of Dr. Joan Decker-Jackson and Dr. Jonathan Berman. The organisms used most have been Leishmania mexicana amazonensis WR 227 and L. donovani WR 130 (Khartoum strain-drug sensitive visceral leishmaniasis). Other organisms presently being cultivated in this laboratory are L. braziliensis WR 424 (Murray isolate from Panama causing cutaneous leishmaniasis), L. braziliensis WR 063 (Terborgh isolate from Peru, causing mucocutaneous leishmaniasis). These organisms are maintained by weekly transfers into Schneider's medium [Grand Island Biological Co., Grand Island, N.Y. (Gibco)] containing 10% heat inactivated fetal bovine serum (HIFBS: GIBCO).

For growing large batches of leishmaniae promastigotes, Brain Heart Infusion Medium (BHI) containing 37 g Difco Brain Heart Infusion/liter water, 10% heat inactivated serum and 26 µg hemin/ml was used. Cells were grown at 26°C in 2000 ml wide Fernback flasks containing 250 ml of BHI and harvested during the exponential growth phase (about day 4).

For defined biochemical experiments the medium of Steiger and Black was used. This medium was used for all transport, uptake and reversal experiments. The cells were depleted of purines by transferring an inoculum from Brain Heart Infusion into Steiger and Black medium with purine omitted, but with 5% heat inactivated fetal bovine serum. The cells were incubated in this medium for 48 hr at 26°C. The cells were then aseptically centrifuged at 5000 x g for 10 min and resuspended to the desired number into fresh Steiger and Black medium Minus purine. By treating the cells in this manner, we avoided as much as possible interference of the metabolism of the compound being tested by the purines in the medium.

RE 1X (Steiger and Black)

Components per liter:

A)	8.0g	NaCL
	400 mg	KCL
	200 mg	$MgSO_4.7H_2O$
	60 mg	$Na_2HPO_4.2H_2O$
	60 mg	KH_2PO_4
	2.0g	glucose
B)	200 mg	L-arginine
	100 mg	L-histidine
	100 mg	L-iosleucine
	300 mg	L-leucine
	250 mg	L-lysine. HCL
	50 mg	L-methionine
	100 mg	L-phenylalanine
	300 mg	L-proline
	400 mg	L-threonine
	50 mg	L-tryptophan
	50 mg	L-tyrosine
	100 mg	L-valine
C)	300 mg	L-glutamine
	1.0 g	$NaHCO_3$
	14.25 g	HEPES (=60 mM)
	20 mg	adenosine
D)	1 mg	D-biotin
	1 mg	choline chloride
	1 mg	folic acid
	2 mg	i-inositol
	1 mg	niacinamide
	1 mg	D-pantothenic acid (hemi-calcium salt)
	1 mg	pyridoxal.HCl
	0.1 mg	riboflavine
	1 mg	thiamine.HCl
E)	2.5 mg	haemin

The experimental design for the testing of promising purine compounds for antileishmanial activity was as follows. The promastigote form of the following organisms were used for testing.

(1) Leishmania mexicana WR #227
(2) Leishmania donovani WR #130
(3) Leishmania braziliensis WR #424

Medium used was the defined medium of Steiger and Black lacking purine, but supplemented with 5% inactivated fetal calf serum and 0.05 mg/ml gentamicin. L. braziliensis does not grow well in this medium at 5% serum, so it was grown 10% serum. The leishmania were initially grown in Brain Heart Infusion and when in log phase ($2x10^6$ cells/ml) these cells were used as an inoculum (0.5ml) and asceptically transferred to 4.5ml of Steiger and Black medium (as described) in 14.5 cm x 1.5 cm test tubes. The compound to be tested was added (at 0.1 - 500uM) and the cells were incubated in a slanted position at 26°C. Optical Density readings at 660nm were taken every 24 hrs. for a total of 96 hrs. Growth experiments were done in duplicate.

Compounds found to be inhibitory were retested in medium containing different purines in order to determine if the inhibitory action could be reversed with the addition of a particular purine. Purines tested for reversal of inhibitory action included: (1) Adenine (2) adenosine (3) guanine (4) guanosine (5) hypoxanthine (6) inosine.

Information gained from purine reversal experiments provides clues as to which purine enzyme or uptake process is being affected. Depending on which purine reverses we then test the anolog in vitro on one of our enzyme systems. For example, if adenine reversed the inhibitory action we would test those enzyme systems which used adenine as a substrate.

Our laboratory is set up to do the following in vitro enzyme assays:

1. Adenine deaminase
2. Adenine phosphoribosyltransferase
3. Guanine deaminase
4. Hxpoxanthine-Guanine phosphoribosyltransferase
5. Nucleosidases
6. Xanthine phosphoribosyltransferase
7. Adenylate deaminase
8. Adenylosuccinate synthetase

Indorporation of ^{14}C-uridine into RNA, ^{14}C-phenylalanine into protein and ^{14}C-thymidine into DNA is determined in order to acertain if macromolecular synthesis is being inhibited.

If we find that no salvage enzyme is inhibited but macromolecular synthesis is, we then test the following enzymes and systems:

1. DNA polymerase
2. RNA polymerase
3. incorporation of compound into RNA through use of mass spectrometry if no isotope is available.
4. inhibition of protein synthesis using a cell-free leishmanial lysate system.

By using the above test systems, we can determine the mode of action of the compound so that this information can be used to (1) synthesize better derivitives (2) explain possible host toxicity and lead to protocols to avoid this complication (3) help understand and combat resistance to the compound.

Compounds (sent by WRAIR) Tested

1) BK 63863 Thiopurinol riboside
2) BK 74731 Oxoformycin
3) BK 86124 Allopurinol riboside
4) BK 86133 5-Azaxanthosine
5) BK 86142 7-Ribosyl-3-deazoguanine
6) BK 63005 3-β-D-Ribofurano sylpyrazolo-[4,3-d] pyrimidine-7-thione
7) BK 48464 6-Aminoallopurinol Riboside
8) BK 95141 3-Ethoxy-6-methylthio-1-β-D-ribofurano sylpyrazolo-[3,4-d] pyrimidine-4(5H)-one
9) BK 95169 3-Ethoxy-1-β-D-ribofuranosylpyrazolo-[3,4-d] pyrimidine-4(5H)-one
10) BK 95187 6-Methylthio-1-β-D-ribofuranosyl-4(5H)-oxopyrazolo-[3,4-d] pyrimidine-3-carboxamide
11) BK 95203 7-Amino-5-chloro-3-β-D-ribofuranosyl-pyrazolo-[4,3-d] pyrimidine (5-chloroformycin)
12) BK 95730 6-Aminoimidazo[4,5-C] pyridin-4(5H)-one (3-Deazaguanine)

Compounds Which Were Significantly Inhibitory at the End of 96 Hrs

Organism	Concentration of BK 63005 (% Inhibition)		
	10 µM	100 µM	500 µM
L. donovani 130	74	82.4	83.8
L. mexicana 227	68.4	83.5	83.0
L. braziliensis 424	34.6	52.9	52.9

Organism	Concentration of BK 74731 (% Inhibition)		
	10 µM	100 µM	500 µM
L. donovani 130	31.8	78.7	84.9
L. mexicana 227	15.5	71.6	83.5
L. braziliensis 424	20.7	36.0	42.2

Organism	Concentration of BK 63863 (% Inhibition)		
	10 µM	100 µM	500 µM
L. donovani 130	60.6	74.3	70.1
L. mexicana 227	58.3	71.9	75.4
L. braziliensis 424	3.1	17.6	24.3

Organism	Concentration of BK 86124 (% Inhibition)		
	10 µM	100 µM	500 µM
L. donovani 130	46.1	50.1	48.7
L. mexicana 227	30.4	41.1	67.9
L. braziliensis 424	1.8	5.8	8.7

Organism	Concentration of BK 48464 (% Inhibition)		
	10 µM	100 µM	500 µM
L. donovani 130	9.8	35.2	56.2
L. mexicana 227	--	13.8	20.4
L. braziliensis 424	--	27.9	41.3

Compounds Which Showed Up To 20% Stimulation

1. BK 86133 5-Azaxanthosine

2. BK 86142 7-Ribosyl-3-Deazoguanine

These compounds appear to be broken down and metabolized as natural purines.

Figures 1-5, show the response of different Leishmania spp. to the most inhibitory purine analogs (sent by WRAIR).

Compounds which were inhibitory were tested for reversal of inhibition by natural purines. The concentration of the analog was 50µM and that of the natural purine was 200 µM. Table 1 shows the ability of natural purines to reverse the inhibition by the analogs. The purines which were most effective in reversing inhibition by the particular analog are as follows:

Purine Anolog	Purine Most Effective in Reversing Inhibition
3-B-D-Ribofurano-sylpyrazolo-[4,3-d] pyrimidine-7-thione BK 63005	Inosine
Oxyformycin BK 74731	Adenosine
Thiopurinol riboside BK 63863	Guanosine (not very effective)
Allopurinol riboside BK 86124	Adenosine
6-Aminoallopurinol riboside BK 48464	Adenosine, Hypoxanthine (equal)
6-Aminoimidazo[4,5-C] pyridin -4(5H)-one (3-deazaguanine) BK95730	Guanosine

Other purine anologs (not sent by WRAIR) have been shown to be very potent growth inhibitors of promastigotes of L. mexicana #227. The following table compares the toxicity of these compounds to some of the most promising anologs sent by WRAIR.

Fig. 1

3-β-Ribofuranosylpyrazolo[4,3-d]-pyrimidin-7-thione

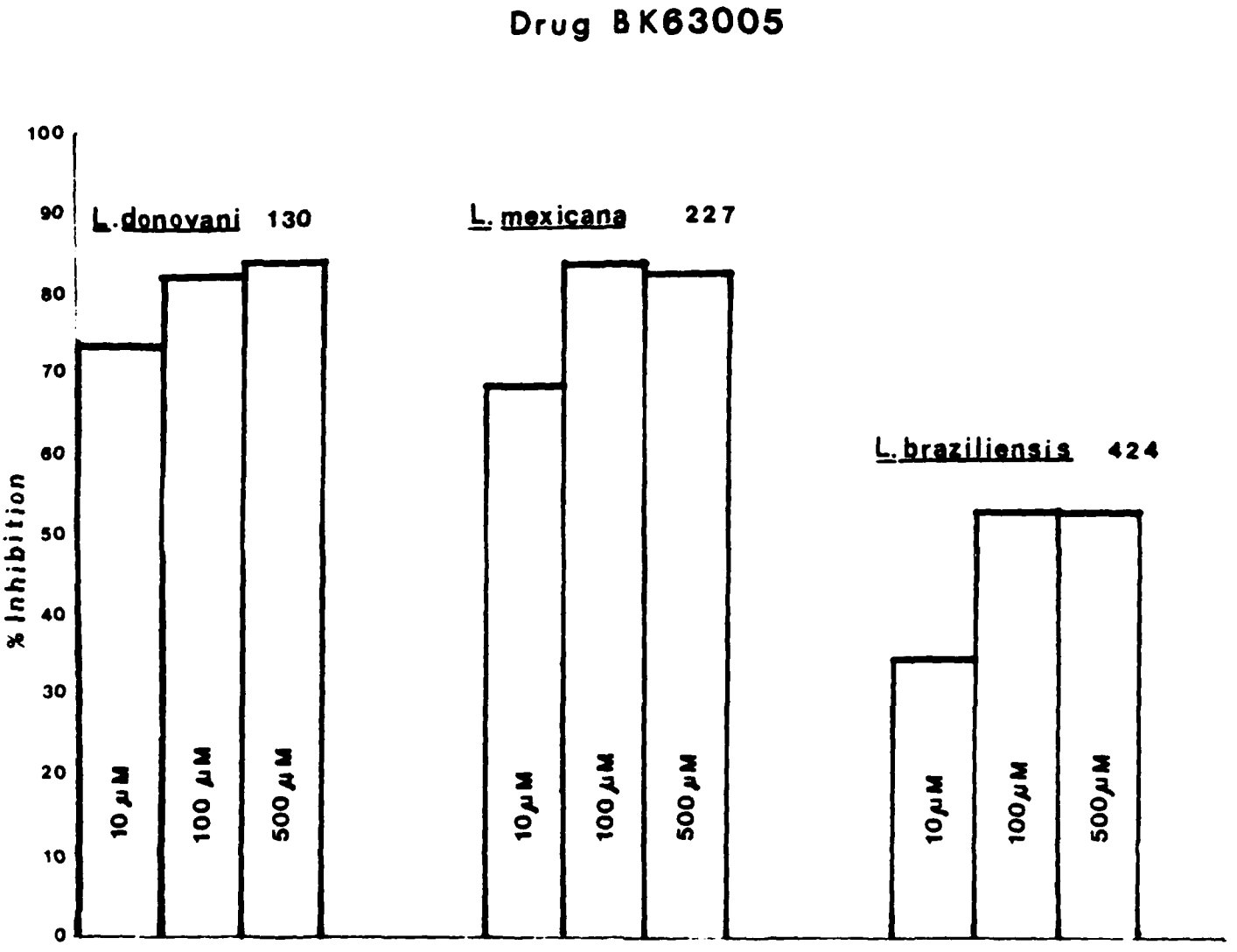


Fig. 2

Thiopurinol riboside

Drug BK63863

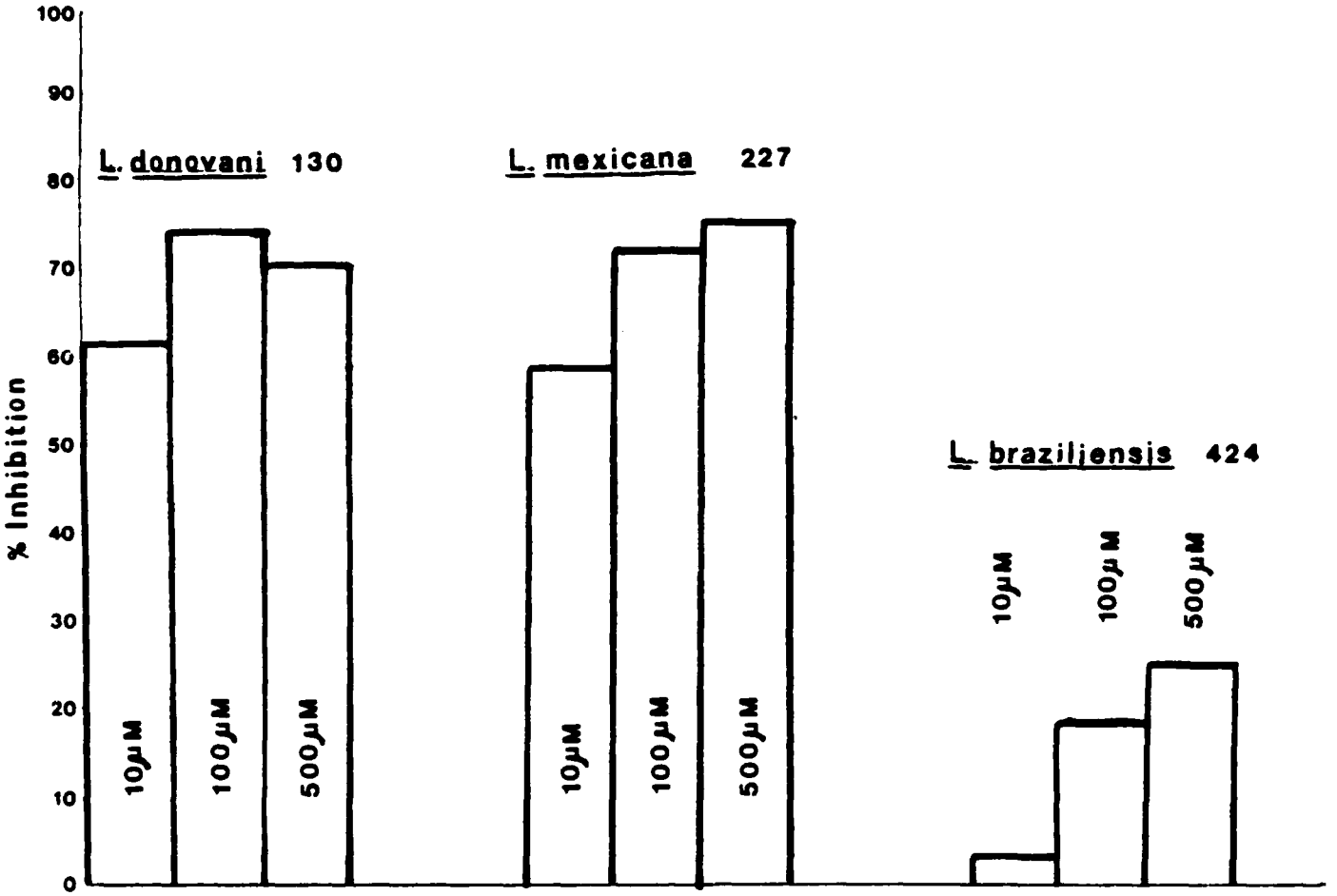


Fig. 3

Oxyformycin

Drug BK74731

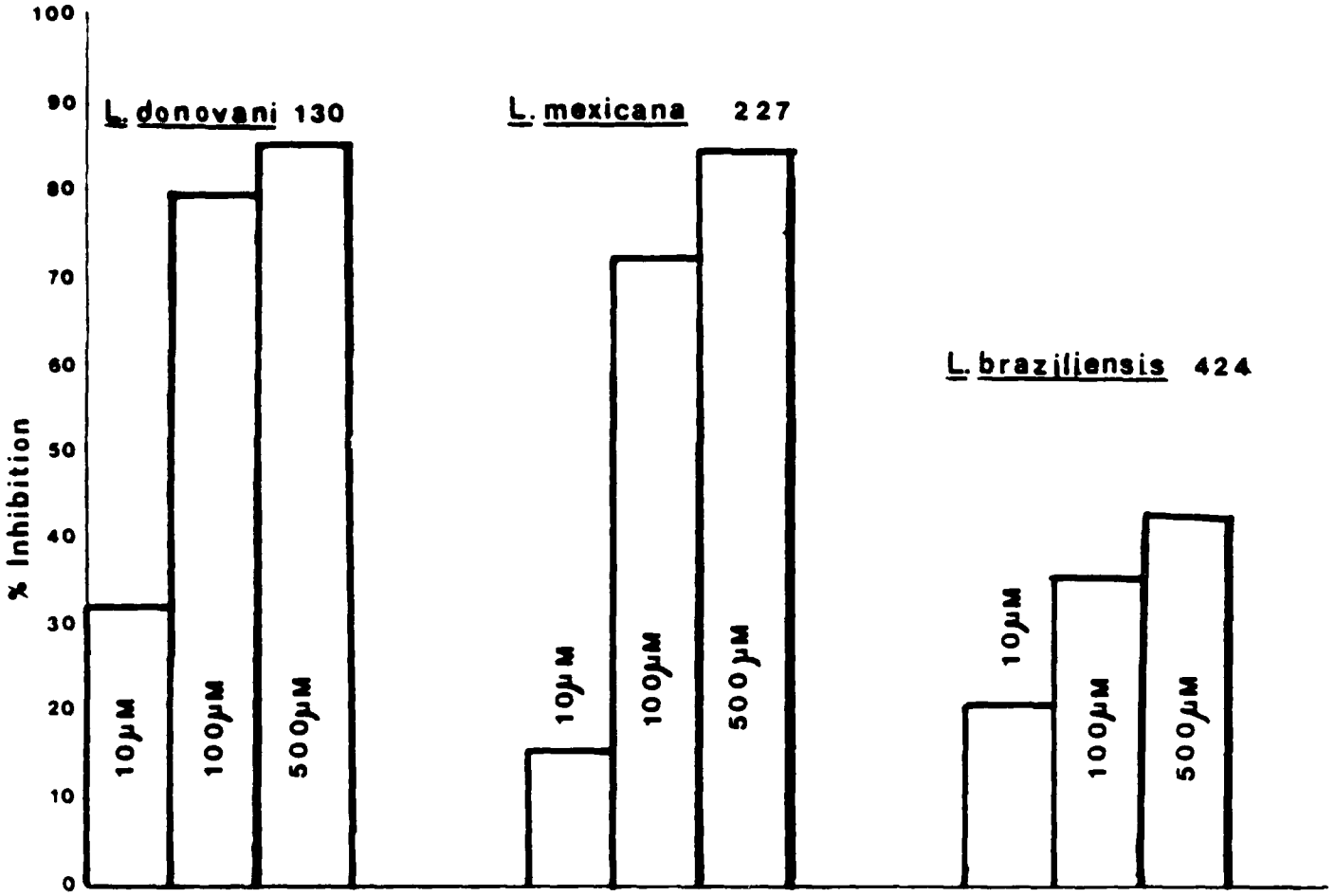


Fig. 4

Allopurinol riboside

Drug BK86124

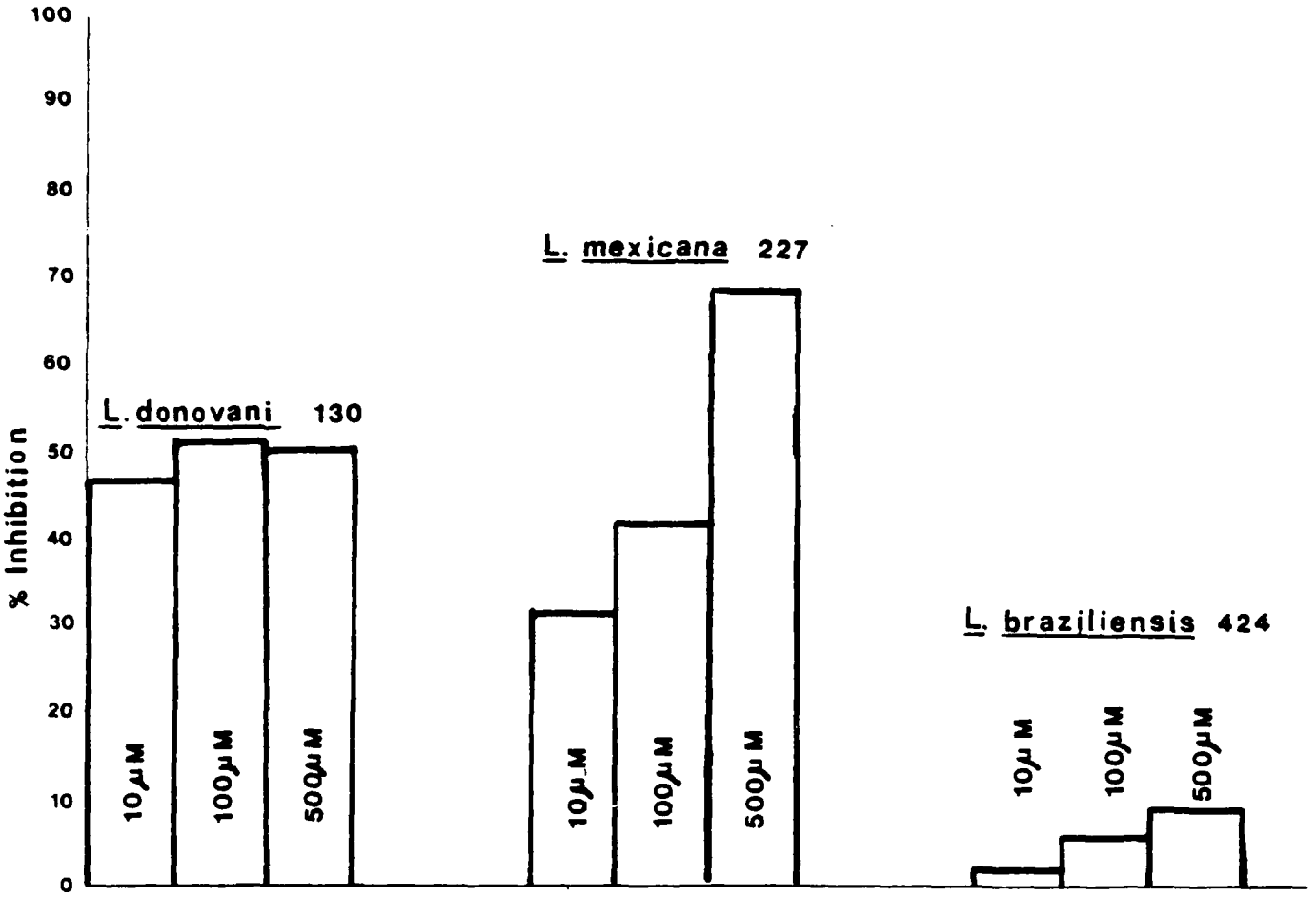


Fig. 5

6-Aminoallopurinol Riboside

Drug BK48464

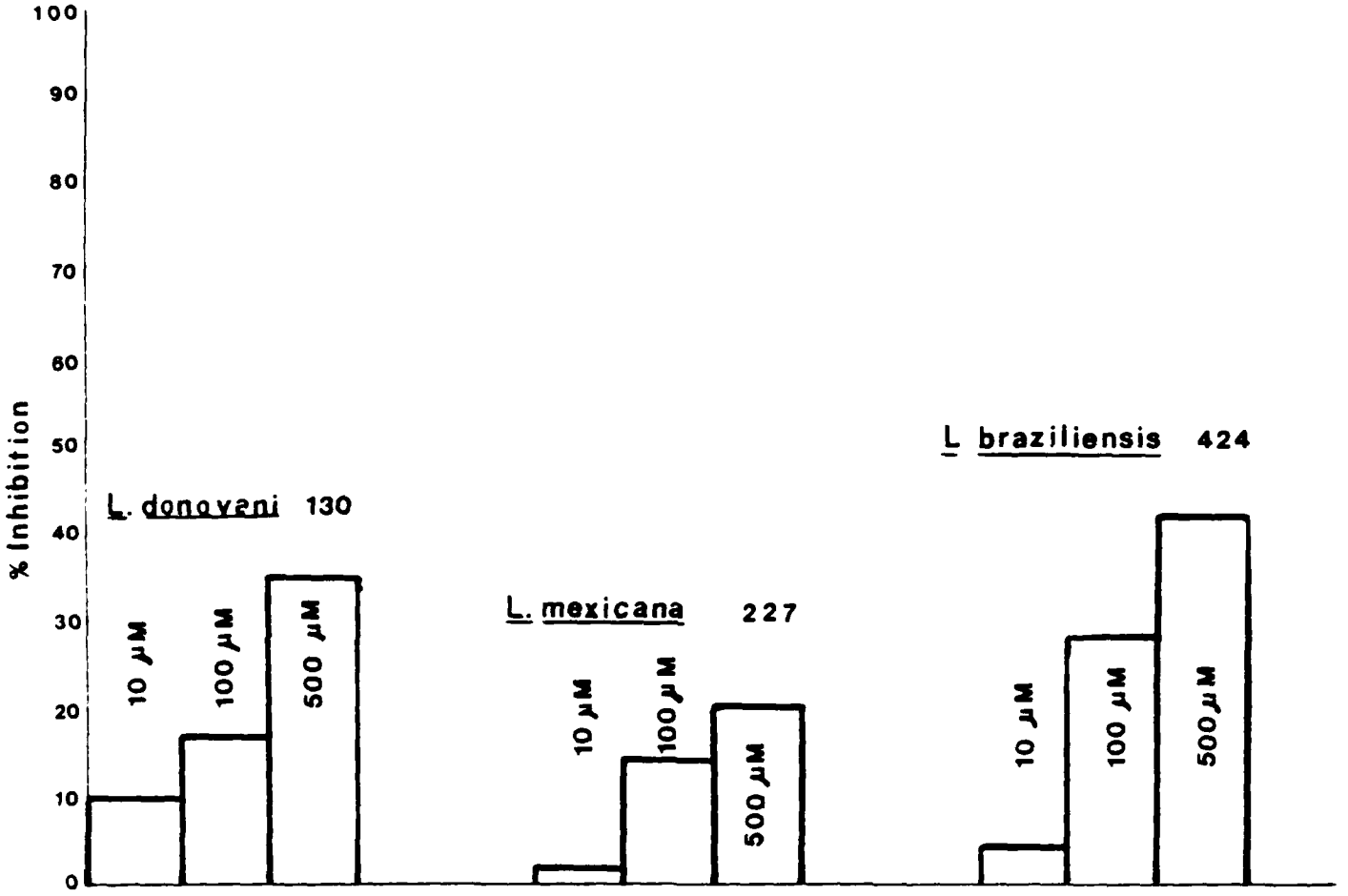


Table 1 Reversal of Analog Inhibition by Natural Purines

Purine Analog 50 μm	% Inhibition Media Only	Purine Added 200 μM Adenosine	Guanosine	Inosine	Hypoxanthine
3-β-D-Ribofurano-sylpyrazolo-[4,3-d]pyrimidin-7-thione BK 63005	70.3	33.6	43.6	29.4	74.5
Oxoformycin B BK 74731	51.7	9.0	25.4	30.0	58.0
Thiopurinol riboside BK 63863	60.5	59.8	51.8	55.9	60.7
Allopurinol riboside BK 86124	29.6	16.6	29.5	16.8	35.6
6-Aminoallopurinol riboside BK 48464	9.9	6.4	18.4	11.3	6.4
6-Aminoimidazo[4,5-C]pyridin-4(5H)-one BK 95730 (3-deazaguanine)	12.7	12.8	None	13.7	8.9

Compound	Concentration Giving 50% Inhibition of Growth of *L. mexicana* #227 Promastigotes
Sinefungin	.005
Formycin B	0.1
Aphidicolin	2
4'Thioadenosine	3
Oxoformycin A	4
Deoxyaristeromycin	8
5-Deoxy-5(isobutylthio)-3-Deazaadenosine (deaza-SIBA)	20
Cordycepin	25
9-Deazainosine	40
Oxoformycin B	50
Allopurinol riboside	200

Tables (2-4) and figs (6-7) show inhibition of these various compounds alone and in combination with others. As can be seen oxoformycin A and B show no additive effect when used in combination. Oxoformycin A is an adenosine analog and oxoformycin B is a xanthosine analog, so it was believed that the toxicity of these compounds would be additive. The fact that oxoformycin B was found to inhibit growth is an important finding, since this compound has been reported to be non toxic to both eukaryotes and prokaryotes (1). It is because of this that oxoformycin B and 9-deazainosine (also proposed to be non-toxic (2) have been combined together and with sinefungin for studies on growth inhibition. Sinefungin is >100X more active in *Leishmania* than mammalian cells, and hits a "hot" target (one which cannot be easily modified or mutated--DNA polymerase). It appears that sinefungin does not have to be metabolized to be toxic, and it is not incorporated into DNA because of its structure. Combining sinefungin at extremely low levels 5-2.5 nM with non toxic compounds which add to the toxicity of sinefungin via another mode of action should provide a safe and rational approach to chemotherapy of leishmaniasis.

Sinefungin is a naturally occurring antifungal antibiotic nucleoside, containing an ornithine residue (3). This compound has been found to display antiparasitic activity against malarial parasites (4), *T. cruzi* (5) and against *Leishmania* spp. (6). We have found that sinefungin inhibits partially purified DNA polymerase activity from *L. mexicana* #227. The preparations we used had been subjected to cell homogenation, centrifugation, and DEAE cellulose chromatography.

Using our partially purified DNA polymerase from *L. mexicana* we have found the K_m for dATP to be 55.5μm (Fig. 8), the K_i for sinefungin to be 15nM (Fig. 9). Sinefungin was found to be a competitive inhibitor of dATP (Figures 10, 11). Fig. 12 shows the effect of increasing concentrations of sinefungin on *L. mexicana* DNA polymerase. Figure 13 shows the inhibition of growth of promastigotes of *L. mexicana* by sinefungin.

It is interesting to note that S-adenosyl-homocysteine (SAH) and S-adenosyl-methionine (SAM) (33 μM) which are similar in structure to sinefungin (Fig. 14) did not inhibit DNA polymerase activity in the above preparation.

Table 2

Compound	Concentration μM	% Inhibition Alone	% Inhibition When Added Together
Oxoformycin A	20μM	78.6	78.0
	30μM	80.9	80.0
	50μM	83.4	82.6
Oxoformycin B	20μM	37.7	
	30μM	46.0	
	50μM	52.8	

% Inhibition determined at 72 hours.

Table 3

Compound	Concentration μM	% Inhibition Alone	% Inhibition When Added Together		
			Concentration (1)		
Allopurinol riboside	50	42.6	61.5		
	100	45.8	61.3		
	200	50.4	57.3		
Oxoformycin B	20	43.2			
			Sinefungin (1)	(2)	Oxoformycin
9-deazainosine	10	35.7	89.4	35.8	59.1
	20	52.6	91.8	48.2	70.4
	40	73.8	93.0	78.3	78.7
	80	85.8	93.8	85.9	87.3
Sinefungin Conc. 1	0.005	66.6			
Conc. 2	0.0025	5.1			
Oxoformycin B(1)	20	43.4			

Table 4

Compound	% Inhibition Alone	% Inhibition with 5 nM Sinefungin	Predicted Inhibition	% Increase in Expected Toxicity
Sinefungin 5nM	19.71	-	-	-
9-Deazainosine 1μM	5.66	19.71	25.66	Less than 5.95
Formycin B 0.1μM	56.71	85.64	76.42	9.22
Oxoformycin 20μM	27.99	80.92	47.70	33.22
Oxoformycin 4μM	66.04	85.53	85.75	Same
Allopurinol riboside 50μM	37.73	61.63	57.44	4.19

% Inhibition was determined at 72 hours.

Fig. 6 INHIBITION OF L. MEXICANA 227 BY PURINE ANALOGS

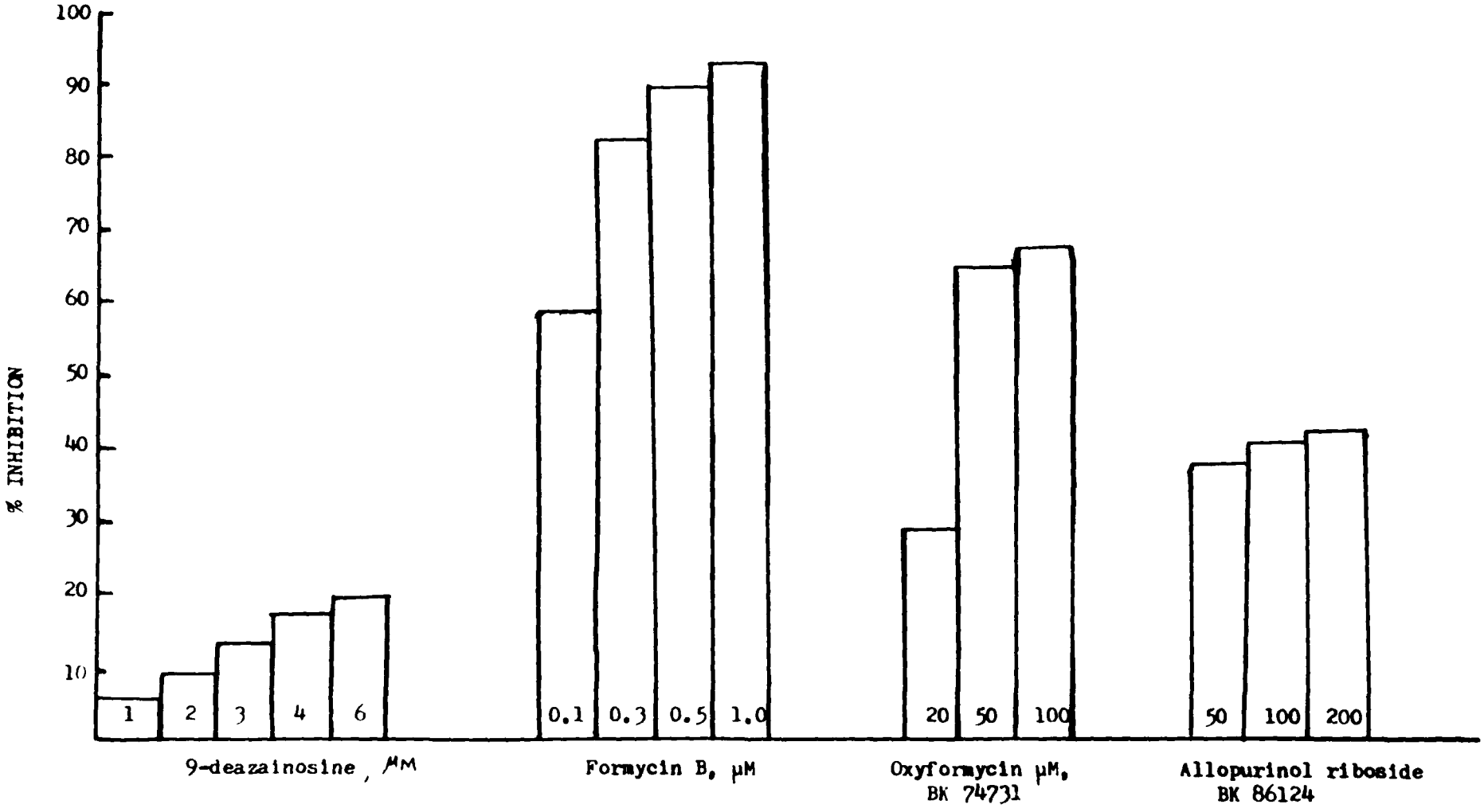


Fig. 7 INHIBITION OF L. MEXICANA 227 by PURINE ANALOGS

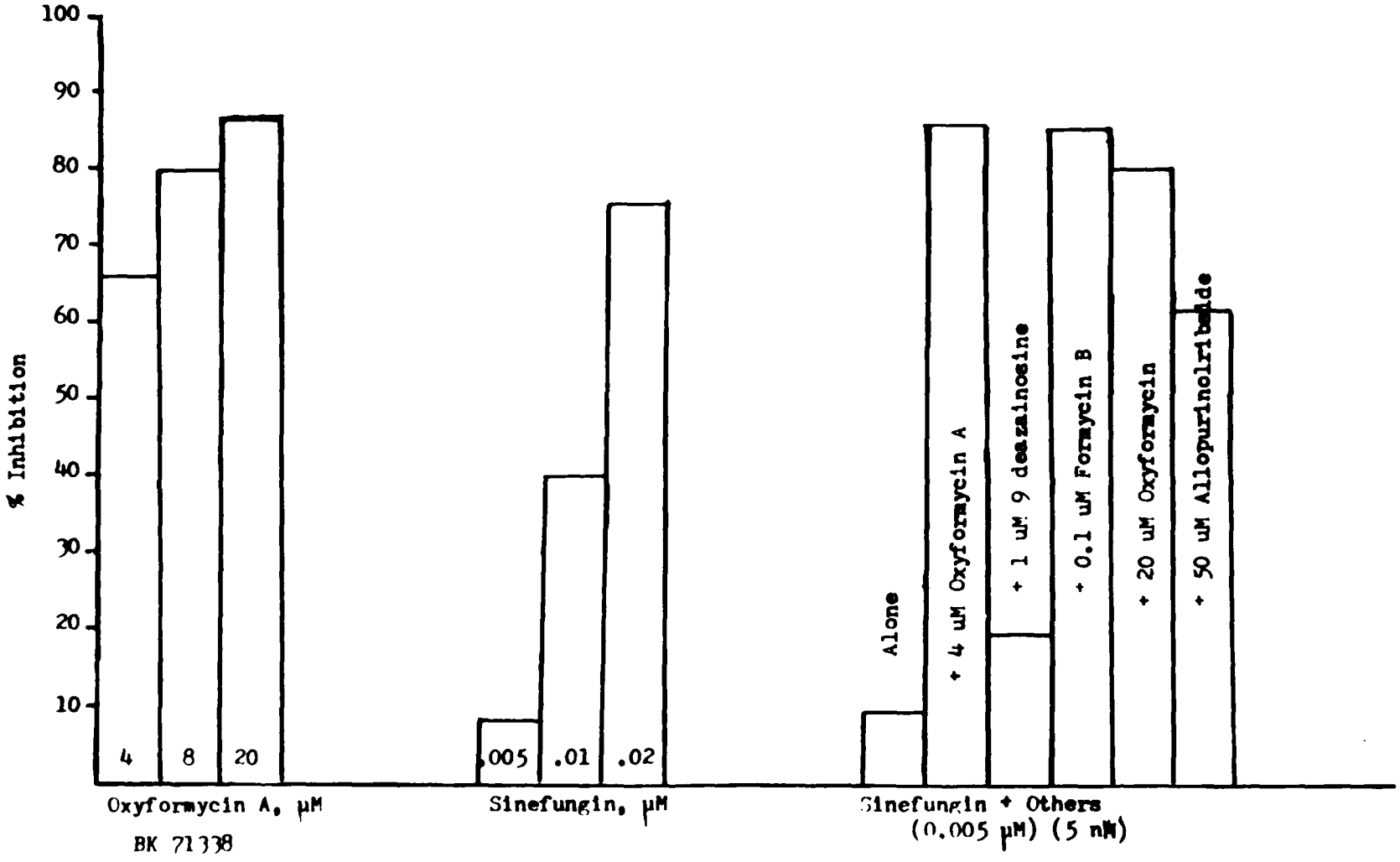


Fig. 8

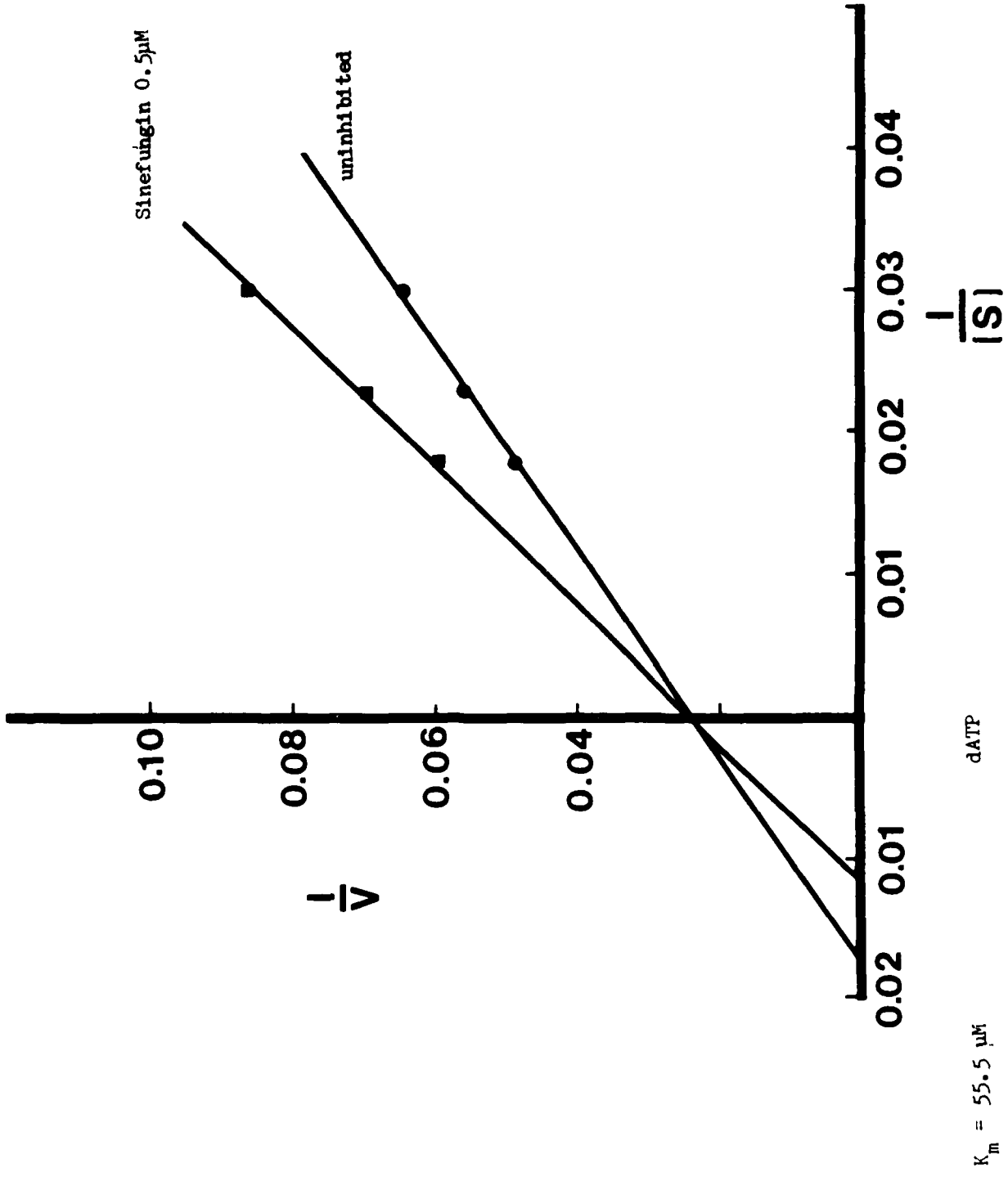
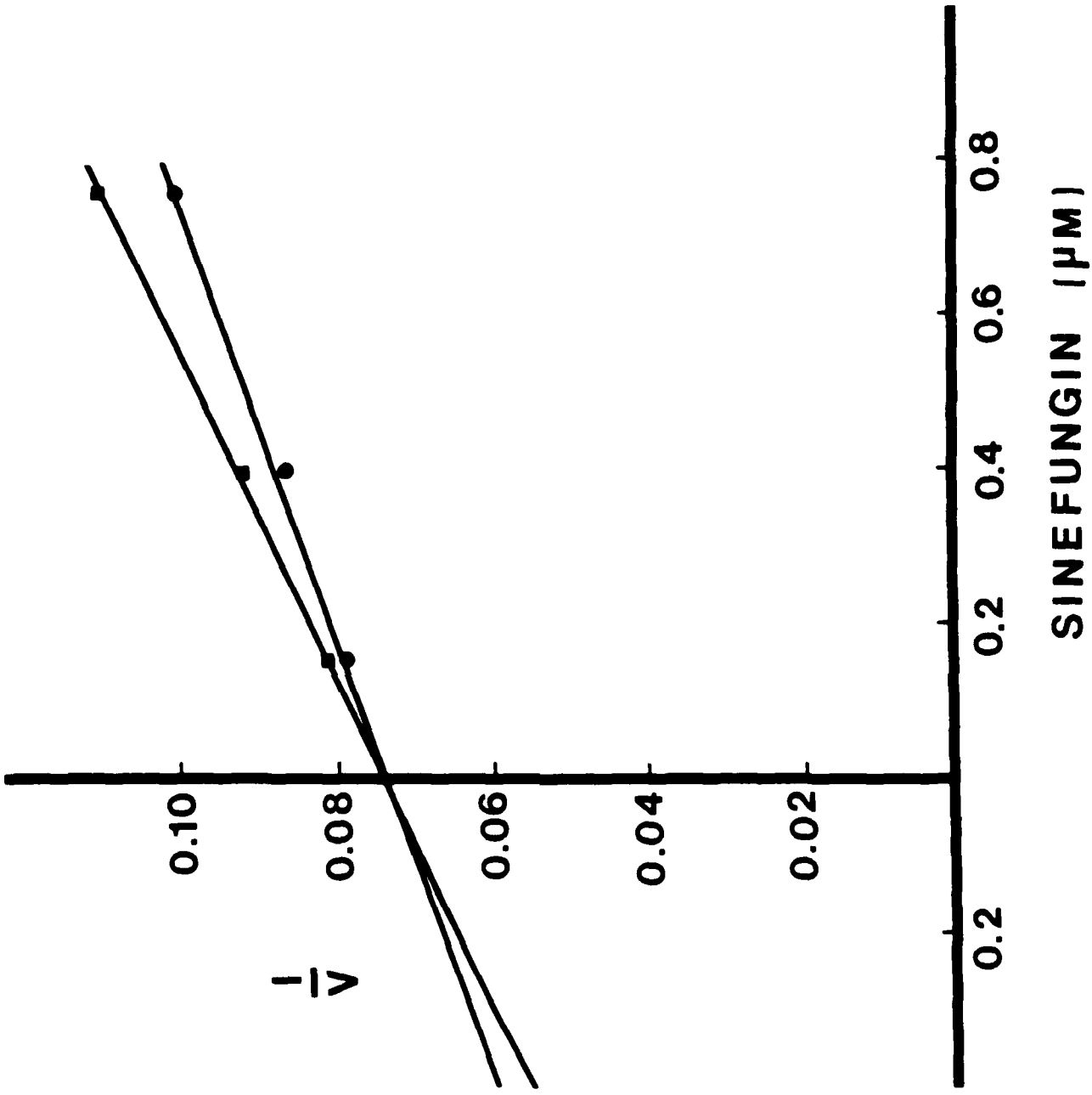
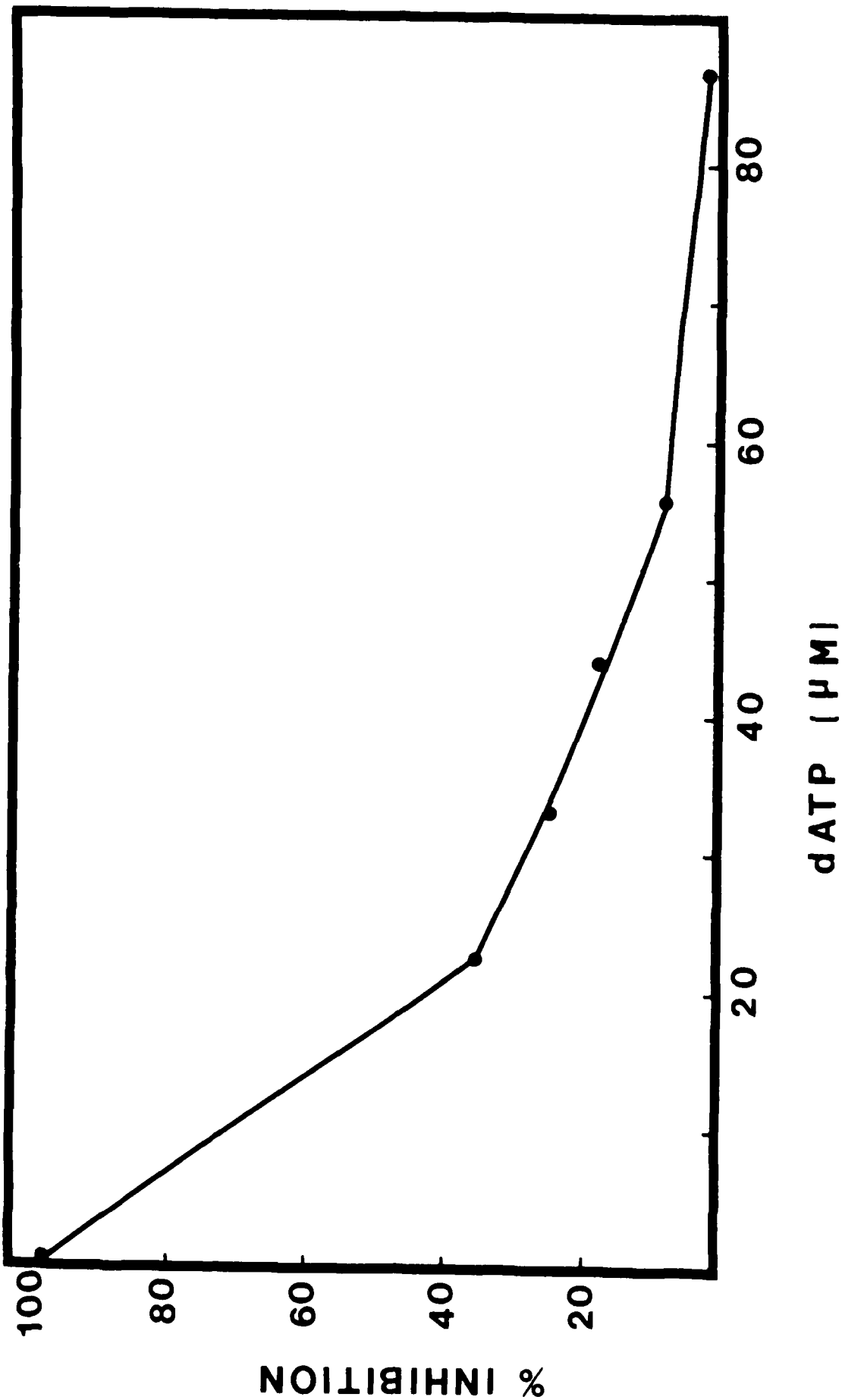

Sinefungin 0.5μM
uninhibited
0.10
0.08
0.06
0.04
0.02
0.01
0.01
0.02
0.03
0.04
1/V
1/[S]
dATP
K_m = 55.5 μM

Fig. 9



K_1 = 15nM sinefungin
S_1 = 44.4 uM dATP
S_2 = 22.2 μM dATP

Fig. 10



Sinefungin 0.5 μM

Reversal of inhibition by dATP

Fig. 11

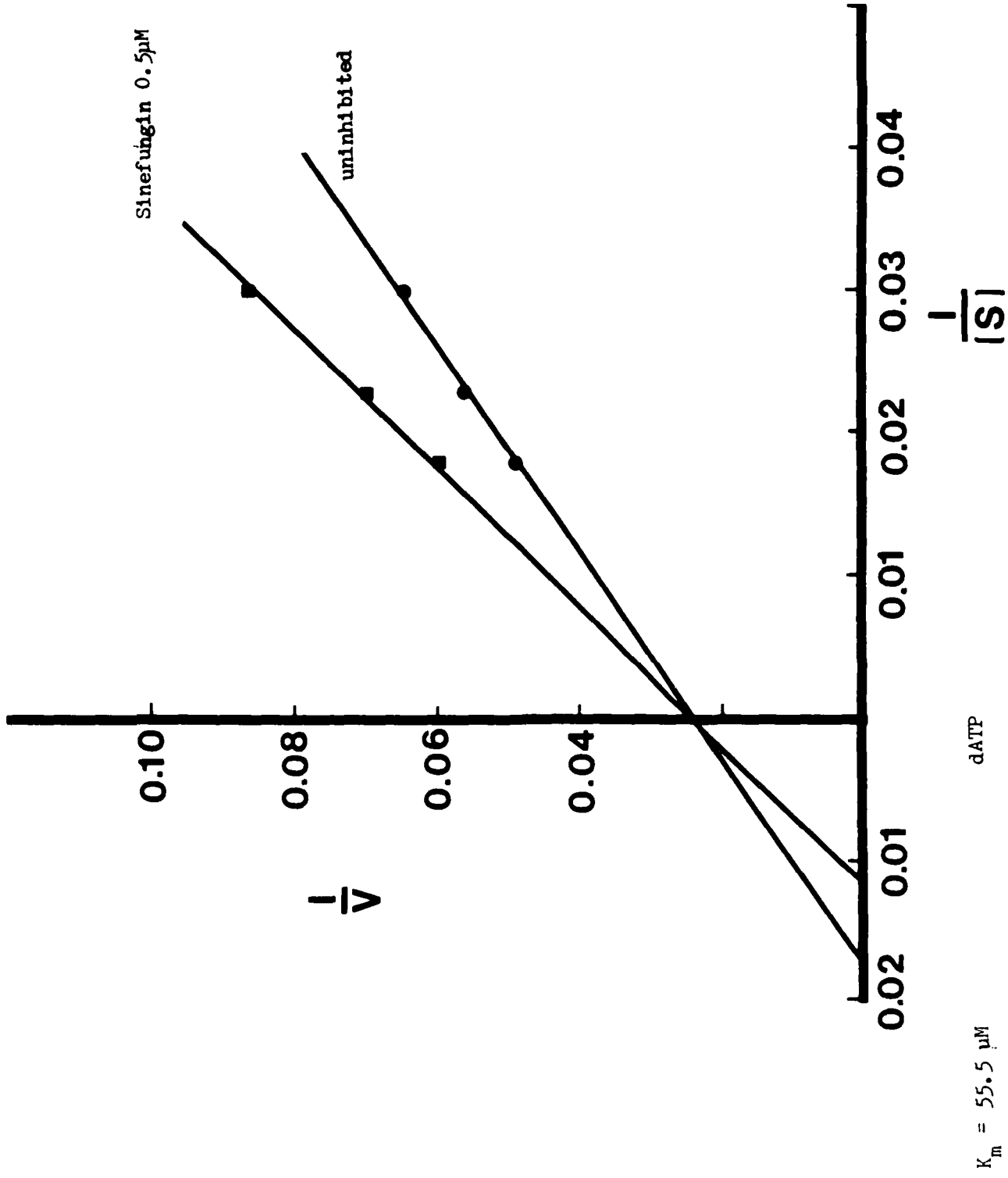

Sinefungin 0.5μM
uninhibited
0.10
0.08
0.06
0.04
1/V
0.02
0.01
0.01
0.02
0.03
0.04
1/[S]
dATP
K_m = 55.5 μM

Fig. 12

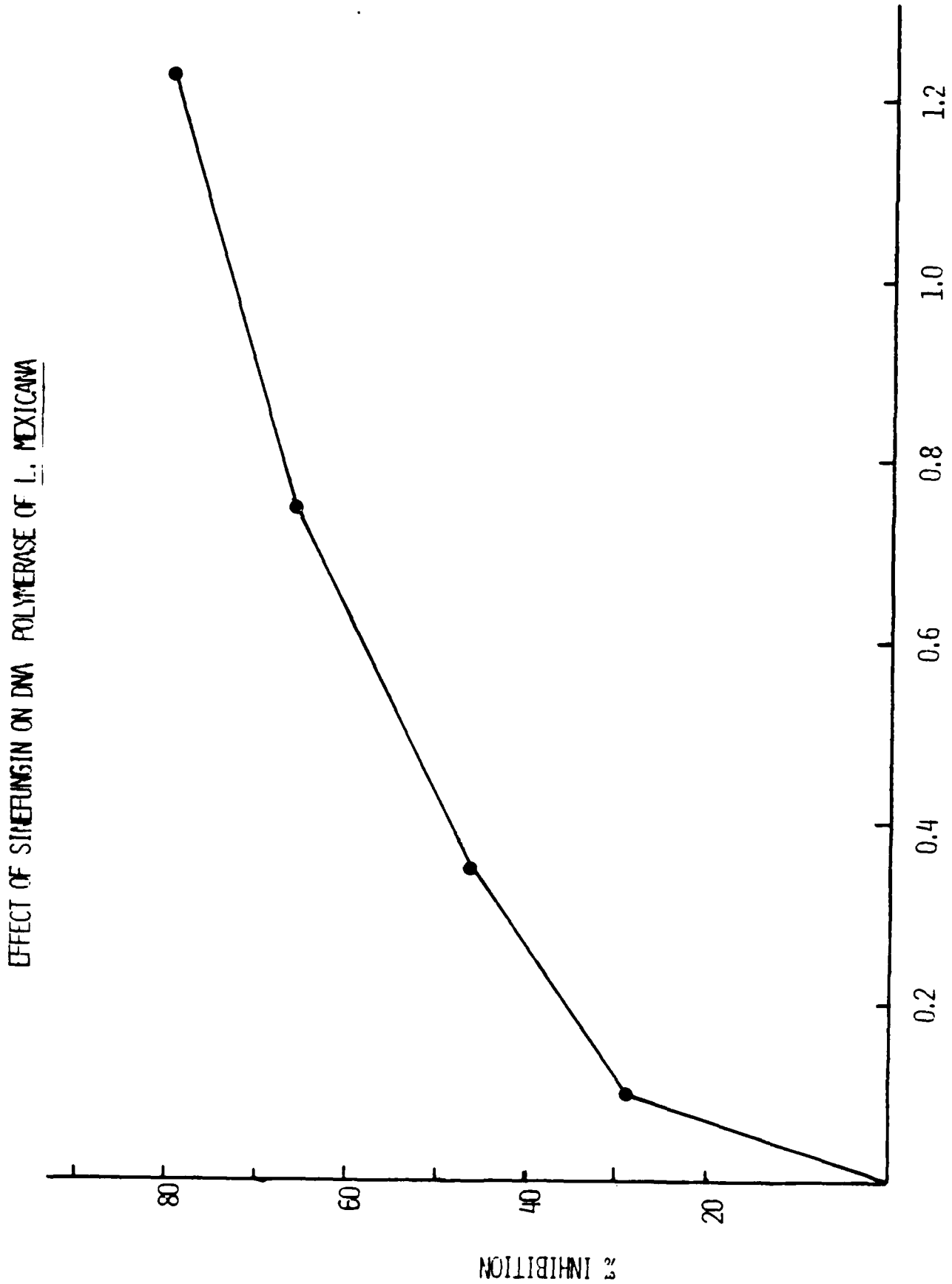

EFFECT OF SINEFUNGIN ON DNA POLYMERASE OF L. MEXICANA
% INHIBITION
20
40
60
80
SINEFUNGIN (μM)
0.2
0.4
0.6
0.8
1.0
1.2

Fig. 13

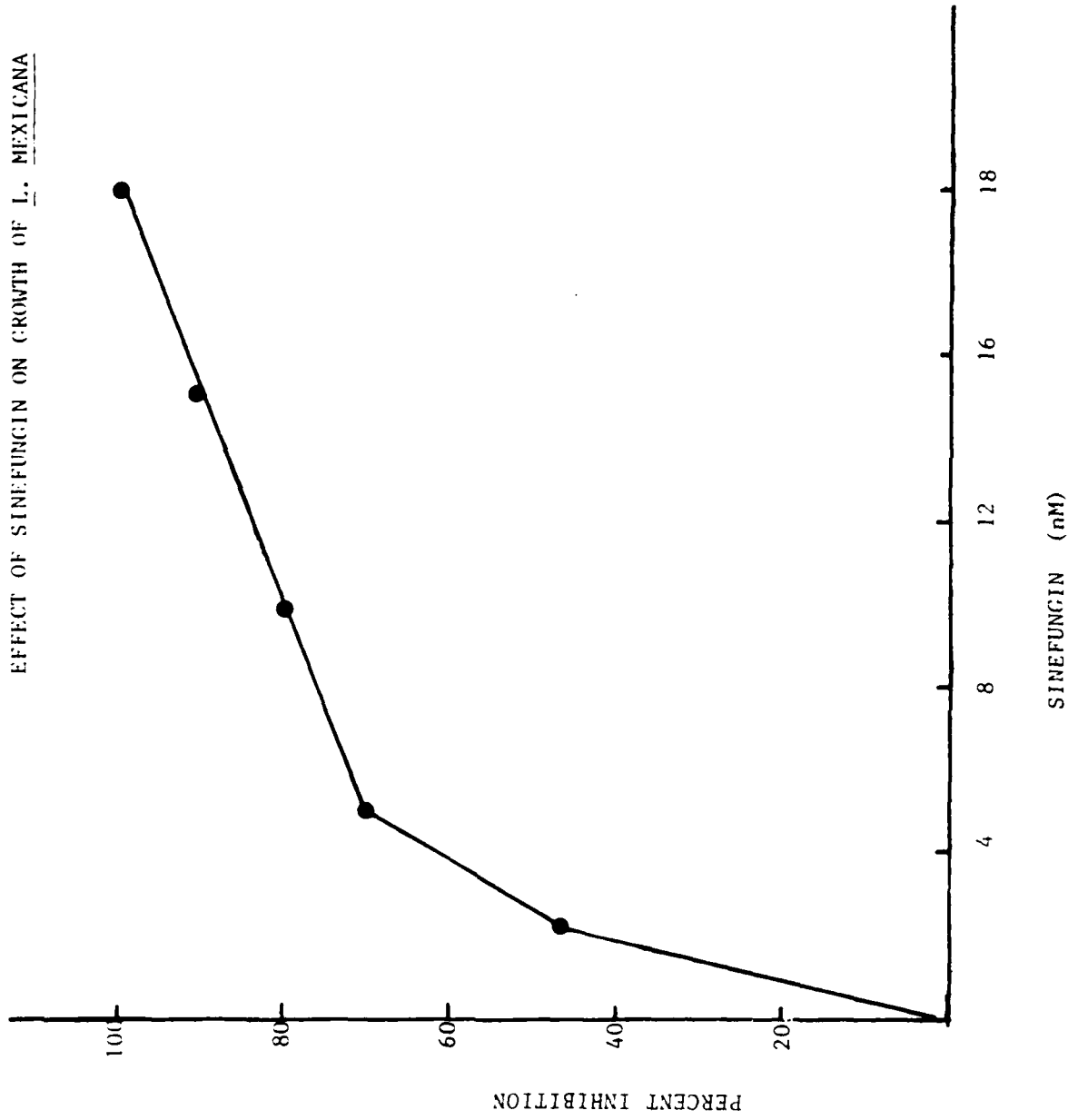
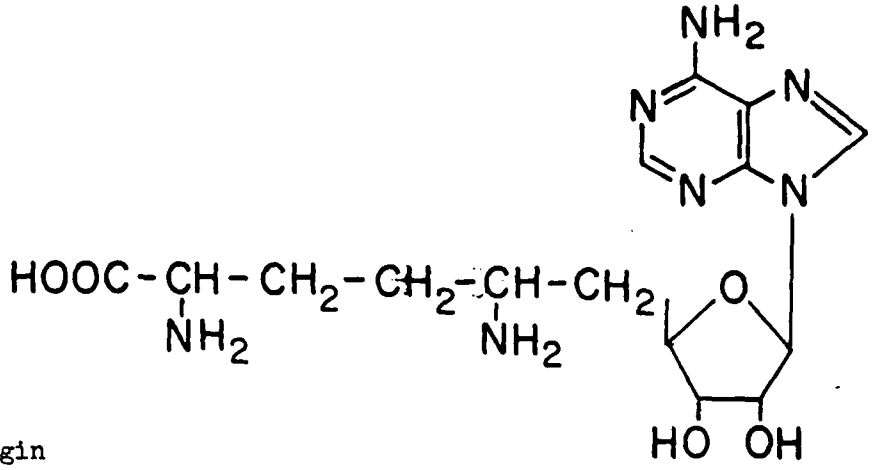
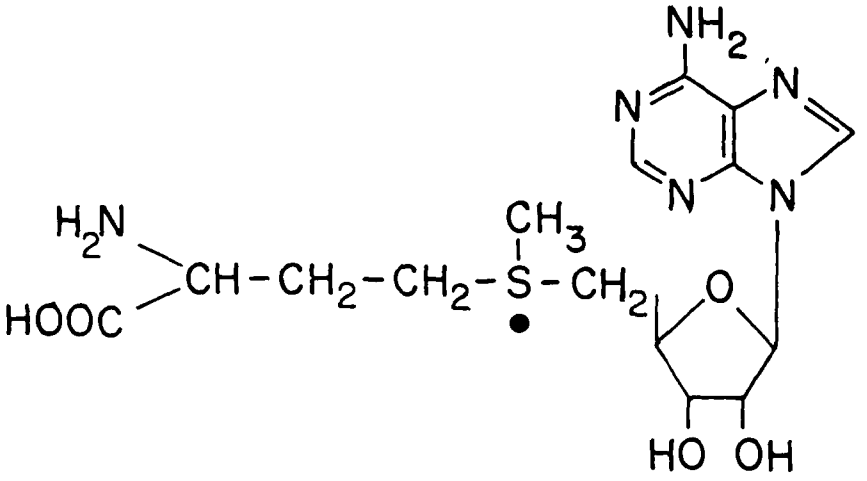

EFFECT OF SINEFUNGIN ON GROWTH OF L. MEXICANA
PERCENT INHIBITION
100
80
60
40
20
4
8
12
16
18
SINEFUNGIN (nM)

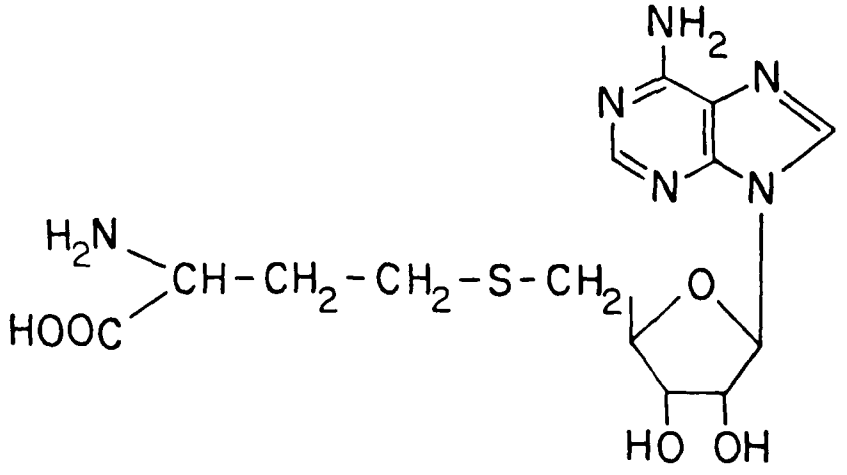
Fig. 14



Sinefungin



SAM



SAH

We developed a purification scheme which resulted in removal of over 99% of the protein with over a 282 fold increase in specific activity (Table 5). The DNA polymerase at this stage of purification was N-ethylmaleimide sensitive and aphidicolin resistant and was not inhibited by sinefungin. These results suggest several possibilities (1) our initial isolation procedures contained an enzyme other than DNA polymerase which was inhibited by sinefungin and subsequently interferred with our assay and (2) during the isolation procedure the DNA polymerase was modified and sinefungin no longer was inhibitory. We know that sinefungin inhibits nuclear DNA synthesis by 80% in vivo experiments. Aphidicolin a DNA polymerase α inhibitor in bacteria, plants and eukaryotes (7), is an extremely potent growth inhibitor of leishmania, but we have not been able to detect a DNA polymerase which is inhibited by this compound. Solari et al., (8) reported that Trypanosoma cruzi DNA polymerase (predominate form) also failed to be inhibited by aphidicolin, but that aphidicolin inhibited growth. It is unclear at present, whether aphidicolin inhibits other crucial metabolic processes or inhibits a DNA polymerase which we presently are unable technically to detect. We are currently determining the mode of action of aphidicolin and sinefungin in more detail.

RNA Polymerase

In experiments to elucidate the mechanism of action of formycin B (FoB), we isolated total RNA from [^{3}H]-FoB exposed cells (8uM for 6 hr) and fractionated this into tRNA, rRNA and mRNA by a sucrose gradient (15-30%). Figure 15, demonstrates that [^{3}H]-FoB metabolites were incorporated into all RNA species. To determine the effect FoB and its metabolites had on RNA polymerase of L. mexicana #227 we partially purified this enzyme.

Isolation and characterization of RNA polymerases in parasitic protozoa will not only help elucidate the mechanism of action of some promising anti-leishmanial agents, but will eventually help us understand the mechanism of promastigote to amostigote transformation.

RNA Polymerase Solubilization. RNA polymerase was solubilized from L. mexicana using procedures similar to those described for other tissues (9-13). Cells (1 liter) were harvested in 250 ml centrifuge bottles and centrifuged at 12,000 rpm for 10 min. The cell pellet was washed twice in Buffer A containing 0.05 M Tris-HCl (pH 7.9, 23°C), 25% (v/v) glycerol, 0.1 mM EDTA, 0.5 mM dithioerythritol, 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride. The cell pellet (usually 4-6 g wet wt.) was suspended in 10-15 ml of Buffer A supplemented with 0.3 M $(NH_4)_2SO_4$ and 1:100 of the following stock solution of protease inhibitors (100x): soybean trypsin inhibitor (4.8 mg ml^{-1}), aprotinin (4.8 mg ml^{-1}) and leupeptin (2 mg ml^{-1}). The cells were lysed by 50 strokes in a Potter-Elvehjem glass-Teflon homogenizer. The suspension was sonicated 6 to 10 times for 10 S with 1 min cooling intervals. This was carried out with a Braun Sonifier 2000 at 4°C. The suspension was centrifuged at 15,000 rpm for 30 min at 4°C. This supernatnat fluid was either subjected to poly (ethylene imine) treatment or to protamine sulfate treatment, dialysis and column chromatography. DNA was removed by adding sufficient 2% protamine sulfate to the crude enzyme to result in a 1:10 dilution. The precipitate from the protamine sulfate step was removed by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was dialyzed in 2 L of Buffer A containing 0.1 M $(NH_4)_2SO_4$. The precipitate that formed after dialysis was removed by centrifugation at 15,000 rpm for 10 min at 4°C.

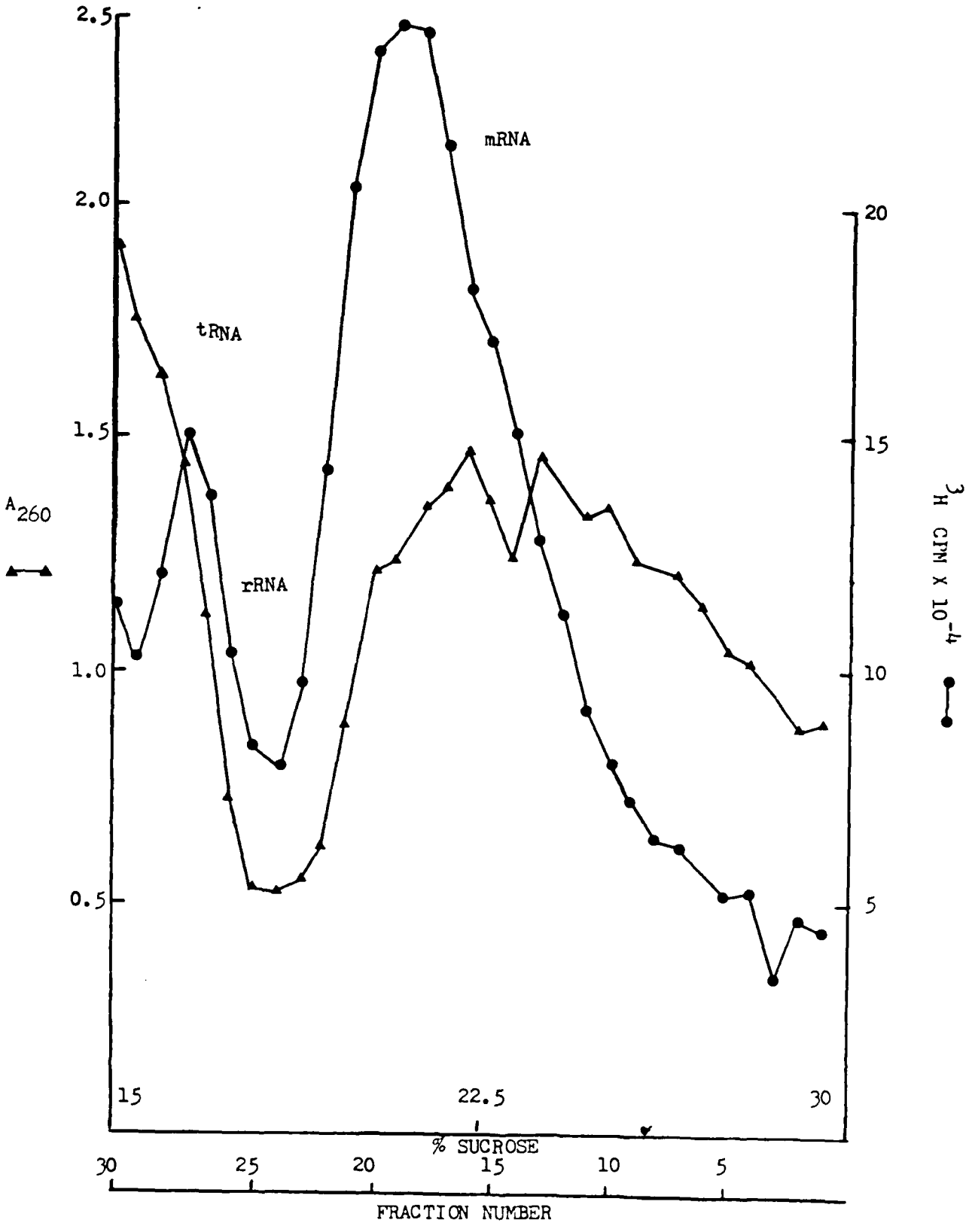
Purification Scheme for DNA Polymerase

Table 5

Purification Step	Total Volume (ml)	Total Protein (mg)	Units $(ml)^{-1}$	Specific Activity (units mg^{-1})	Purification (-fold)
Lysed Cells	25.4	1,636.27	42.42	0.66	-
Crude extract (15,000 rpm, 1 hr.)	22.8	1,350.90	41.95	0.70	1.08
DEAE cellulose	25.2	1,064.70	36.81	0.87	1.32
Heparin-Sepharose	16.5	102.46	255.24	41.10	62.3
DNA cellulose	4.2	5.56	245.00	185.68	285.66

Fig. 15

SUCROSE GRADIENT SEPARATING RNAs FROM TOTAL RNA



Heparin-Sepharose CL-6B (Pharmacia Fine Chemical Co.) was equilibrated with six column volumes of Buffer A contaning 0.1 M $(NH_4)_2SO_4$. From 4-7 ml of the dialyzed crude extract was applied to the column and 1 ml fractions were collected. After the column was washed with two volumes of the above buffer and no further protein was eluted from the column, RNA polymerase was eluted with Buffer A containing 0.5 M $(NH_4)_2SO_4$. Peak fractions were pooled and dialyzed overnight in 2L of Buffer A containing 0.075 M $(NH_4)_2SO_4$.

DEAE-Sephadex A25. The gel was prepared by suspending 8g of the powder into 100 ml of 0.05 M Tris HCl (pH 7.9), 0.1 mM EDTA and 1 M $(NH_4)_2SO_4$. The gel was swelled by heating in a 90°C water bath for 1.5 hr. The gel was cooled overnight to room temperature and then heated again to 70°C. When the gel slurry reached room temperature the buffer was decanted, and the above buffer with 0.075 M $(NH_4)_2SO_4$ was added. The gel was poured into a 1.5 x 17 cm column and equilibrated with several column volumes of Buffer A containing 0.075 M $(NH_4)_2SO_4$.

The dialyzed fractions from the Heparin-Sepharose chromatography were applied. Buffer A (60 ml) containing 0.075 M $(NH_4)_2SO_4$ was washed through the column. The RNA polymerase was then eluted with a linear gradient 0.075-0.5 M $(NH_4)_2SO_4$ in a volume of 100 ml. Active fractions were pooled and dialyzed overnight in 2 L of Buffer A containing 0.05 M $(NH_4)_2SO_4$.

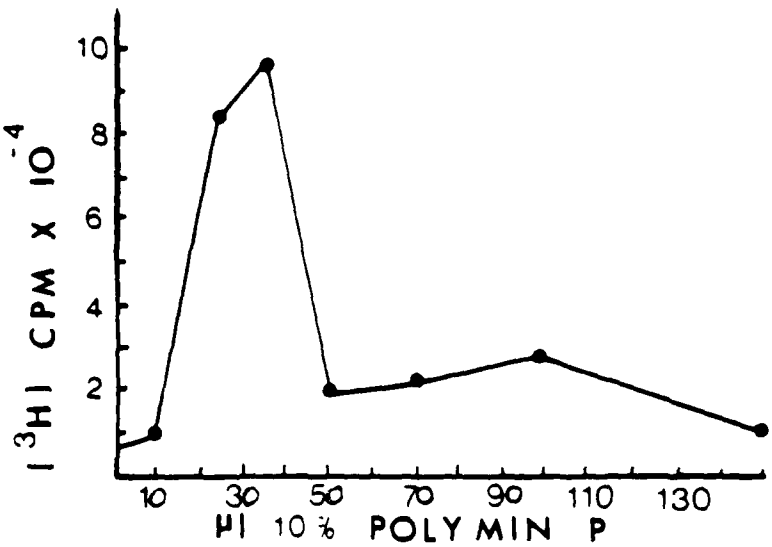
Carboxymethyl-Sephadex Chromatography. The gel was prepared as described for DEAE Sephadex chromatography except that the column was equilibrated with Buffer A containing 0.05 M $(NH_4)_2SO_4$. The pooled material from the DEAE-Sephadex column was applied and the enzyme was eluted in a 0.05-0.3 M $(NH_4)_2SO_4$ linear gradient.

Sephacryl S-300 Chromatography. The gel was equilibrated in Buffer A without glycerol, and poured into a 1.5 x 100cm column. The packed column measured 1.5 x 75cm. RNA polymerase obtained from DEAE chromatography was subjected to gel filtration on Sephacryl S-300 for a molecular weight determination. Since RNA polymerase is very unstable in the absence of glycerol, 0.1 ml of glycerol was placed in the tubes collecting the fractions (1 ml). The resulting glycerol concentration in the fractions collected was 10%.

Poly (ethylene imine) Precipitation. A 10% (v/v) solution of poly (ethylene imine) was prepared and neutralized as described (14). In order to determine the optimal conditions for the use of this method of purification, crude extract was divided into 1 ml fractions and each of these was mixed with the indicated volumes (Fig. 16) of 10% poly (ethylene imine) and allowed to stand 15 min so that complete precipitation could take place. These fractions were then centrifuged in a Fisher microfuge for 10 min at 7,500 x g. The supernatant fluid from each fraction was collected and assayed for RNA polymerase activity.

RNA Polymerase Assay. Assays for monitoring column fractions were conducted in 96 well microplates. When drug studies were being performed, assays were conducted in 1.5 ml microcentrifuge tubes to prevent protein or drug from sticking to the surface. The radioactive assay used was a modification of that used by Roeder (10). The following components were present in each tube in a final volume of 51 µl: 0.05 M Tris HCl (pH 7.0); 2 mM $MnCl_2$; 0.6 mM ATP; 0.6 mM GTP; 0.6 mM CTP; 0.05 mM UTP; 0.088 mg/ml denatured calf thymus DNA;

Fig. 16



and 0.2 μ Ci [^{3}H] UTP. Unless otherwise stated the $(NH_4)_2SO_4$ concentration was 0.1 M. Each reaction was begun with the addition of 20 μl of enzyme solution in Buffer A. After 20 min at 30°C, reactions were terminated by pipetting 10 μl of a solution containing 2.5% SDS and 0.15 M sodium pyrophosphate (14). The reaction mixture was then pipetted onto DEAE-cellulose discs (Whatman DE81) followed by immersion in 0.5 M Na_2HPO_4. The filters were washed six times for 5 min each in this buffer, twice in distilled H_2O, twice in 95% EtOH, once in ether, and air-dried (10). The discs were counted in Fisher Scinti Verse II in a Beckman scintillation counter.

One unit of activity is defined as the incorporation of 1 pmole of UMP into RNA in 20 min under standard assay conditions (10).

Polyacrylamide Gel Electrophoresis. RNA polymerase which had been chromatographed on Heparin-Sepharose, DEAE-Sephadex, and Phosphocellulose was subjected to non-denaturing polyacrylamide gel (7%) electrophoresis using the method of Davis (14). One tube was sliced into 4 mm sections immediately after electrophoresis and the slices were assayed in 3 times the standard assay volume. The slices were incubated 90 min at 30°C, and the reaction mixture was then spotted on DE81 cellulose pads and prepared for scintillation counting as described above. The other gels were stained overnight in 0.2% comassie blue R-250, 50% methanol and 10% acetic acid. The stained gel was destained in a Bio-Rad destainer utilizing 25% methanol and 10% acetic acid.

Protein Assays. Protein concentrations were either determined by the dye-binding method of Bio-Rad Labs (595 nm) or by a modification of the Bio-Rad Labs method. The modified method was performed in 96 well microplates by adding 80 μl of Bio-Rad dye and 20 μl of a column fraction. The plate was then read in a Dynetech 580 microplate reader at 575 nm.

Denatured DNA. Calf thymus DNA (Sigma, 0.5 mg/ml) was heated in a water bath at 90°C for 5 min and then quickly chilled on ice.

RESULTS

RNA polymerase activity could not be detected in crude preparations of L. mexicana. It appears that the removal of DNA and inhibitory proteins are necessary to detect this enzyme. In our experiments 96-99% of the protein concentration was removed from the initial preparation as well as the DNA before RNA polymerase could be detected. The method utilizing poly (ethylene imine) precipitation provides a rapid procedure for removing inhibitory substances. Poly (ethylene imine) is very basic and is expected to precipitate acidic molecules including nucleic acids, nucleoproteins (chromatin and ribosomes), and acidic proteins (including RNA polymerase II) (11). The results of precipitating RNA polymerase with a 10% poly (ethylene imine) solution are shown in Fig. 16. The enzyme activity in the supernatant initially increases, reaches a maximum and then decreases. The initial increase results from the removal of one or more substances which inhibit RNA polymerase. The subsequent decrease in activity reflects the coprecipitation of the enzyme with the poly (ethylene imine), as has shown to be the case with other systems (11). Fig. 16 shows that all the RNA polymerase activity is effectively precipitated at any concentration of poly (ethylene imine) above 0.4% (v/v) crude extract. Addition of 30 μl of a 10% poly (ethylene imine) solution ml^{-1} of crude extract removed 97-99% of the protein and nucleic acids.

The supernatant fluid from the poly (ethylene imine) precipitation was subjected to DEAE-Sephadex chromatography and yielded only one active RNA polymerase peak. Also, the method of Smith and Braun (13) utilizing poly (ethylene imine) precipitation of RNA polymerase activity and subsequent release of RNA polymerase activity with $(NH_4)_2SO_4$ failed to detect more than one RNA polymerase when chromatographed on DEAE-Sephadex. Chromatography on DEAE-Sephadex appears to be the most effective means for resolution of the various enzymes in a single chromatographic step. RNA polymerases I, II and III from most cell types are eluted from this ion-exchange matrix at concentrations of approximately 0.05-0.15, 0.15-0.25 and 0.20-0.35 M ammonium sulfate respectively (16). Since we wanted a method which would allow us to detect all three polymerases poly (ethylene imine) precipitation was abandoned. It appeared that this method was inadequate for the solubilization of all RNA polymerase types in L. mexicana promastigotes.

To determine the effect of sonication on releasing RNA polymerase activity, we sonicated the crude homogenate for different lenghts of time, precipitated inhibitory material with 30 µl poly (ethylene imine) ml^{-1} of crude homogenate, centrifuged (7,500 x g for 5 min), and assayed for RNA polymerase activity. Fig. 17 demonstrates that sonication is necessary to release RNA polymerase activity, but once the enzyme(s) are released, they are inactivated by further sonication.

We found chromatography on Heparin-Sepharose after homogenation, sonication and protamine sulfate treatment of the crude extract to be a very efficient step in the purification of RNA polymerase. The protein concentration was reduced by 95%. RNA polymerase was eluted with a 0.5 M $(NH_4)_2SO_4$ step gradient in Buffer A. The inhibitory substances were removed and one peak of RNA polymerase activity was detected (Fig. 18). Pooling of active fractions from the Heparin-Sepharose and subsequent chromatography on DEAE-Sephadex increased the specific activity 66 fold (Fig. 19). Further chromatography on Carboxymethyl-Sephadex (CM) of pooled active fractions resulted in a 2.25 fold increase in specific activity from the previous step (Fig. 20). Table 6 shows a purification scheme for RNA polymerase. During Heparin-Sepharose and CM-Sephadex chromatography only one RNA polymerase peak was observed. Several times during DEAE-Sephadex chromatography a small RNA polymerase peak could be detected closely associated with the major RNA polymerase peak. Assay of each of the peak fractions, revealed similar sensitivities to α-amanitin and $(NH_4)_2SO_4$ concentrations. It is not known whether the appearance of this second peak is the result of proteolysis of the enzyme or represents a multiple form of an α-amanitin-sensitive RNA polymerase.

The results of gel electrophoresis under nondenaturing conditions of RNA polymerase which had been subjected to protamine sulfate treatment, Heparin-Sepharose, DEAE-Sephadex, and phosphocellulose chromatography is shown in Fig. 21. Only two protein bands could be detected when stained with commassie blue. RNA polymerase activity could not be detected when gels were sliced and assayed immediately after electrophoresis. Gel filtration on Sephacryl S-300 of RNA polymerase pooled from a CM-Sephadex column revealed a molecuaar weight of 360,000 (Fig. 22-23). Since this column did not contain glycerol which stabilizes the polymerase the specific activity after gel filtration was very low. Gel filtration with 25% glycerol in Buffer A takes almost 48 hr to complete. Because of this, the glycerol was omitted, but added to the fractions after filtration.

Fig. 17

RNA POLYMERASE ACTIVITY

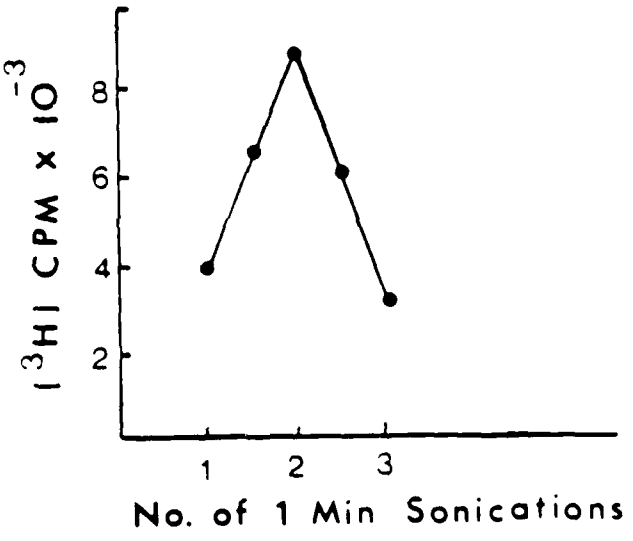
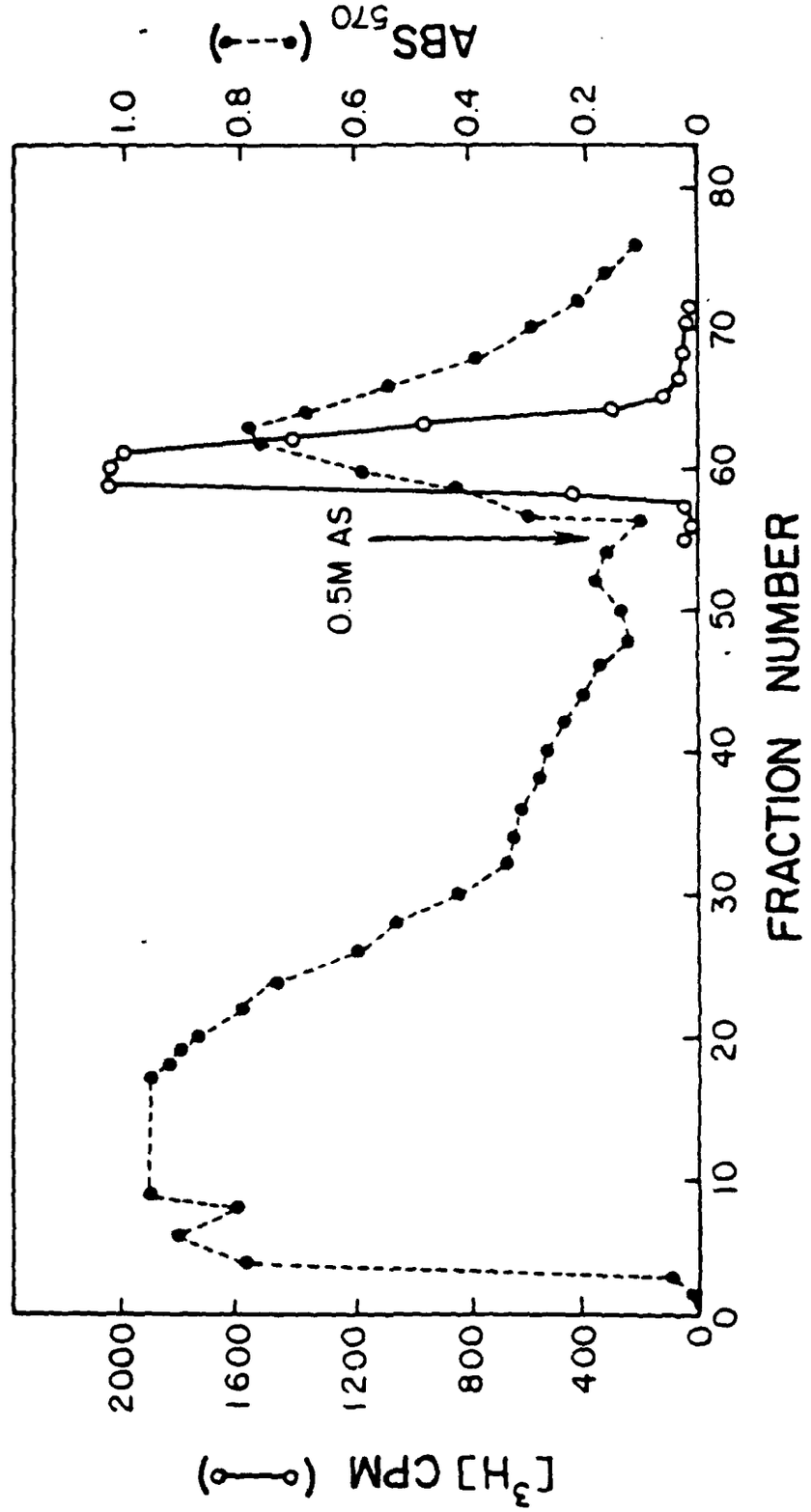


Fig. 19



Heparin-Sepharose Chromatography of RNA Polymerase

Fig. 1

DEAE-Cellulose Chromatography of RNA Polymerase

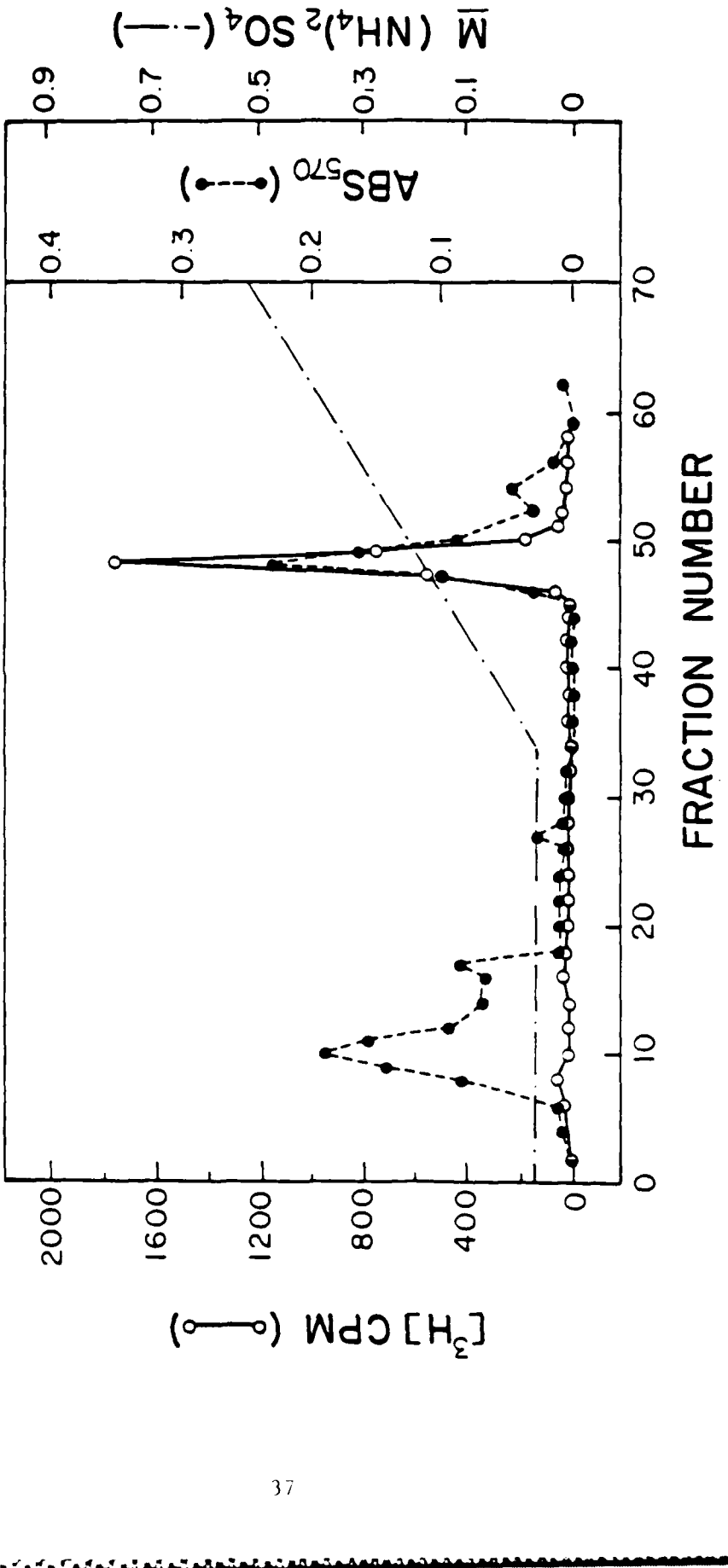
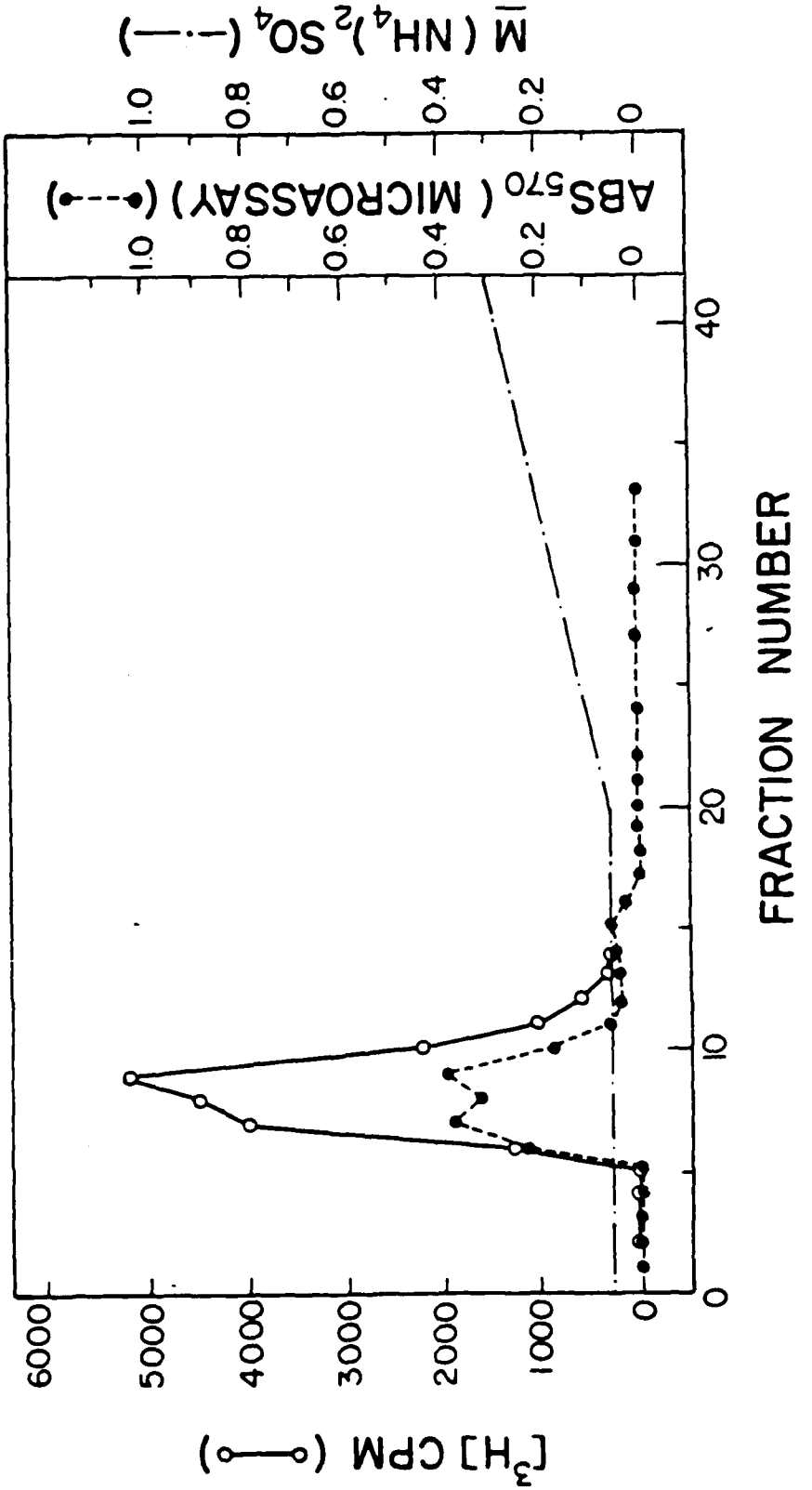


Fig. 2?



Carboxymethyl-Sephadex Chromatography of RNA Polymerase

Table 6. Purification scheme for RNA-polymerase III

Purification Step	Total Volume (ml)	Total Protein (mg)	Units* $(ml)^{-1}$	Total Activity (units)	Specific Activity $(units\ mg^{-1})$
Whole sonicate	19.3	768	Non detectable	-	-
Crude extract (15,000 rpm, 30 min)	15.0	498	Non detectable	-	-
Protoamine sulfate treatment	16.3	453	Non detectable	-	-
Heparin-Sepharose	6.7	23	2.1	14.1	0.6
DEAE-Sephadex	6.0	2.4	15.8	94.8	39.5
CM Sephadex	8.5	1.7	17.7	150.5	88.5

*One unit of activity is defined as the incorporation of 1 pmole of UMP into RNA in 20 min under standard assay conditions. Assay conditions are described under Methods.

Fig. 21

Polyacrylamide Gel Electrophoresis Under Nondenaturing Conditions of RNA Polymerase

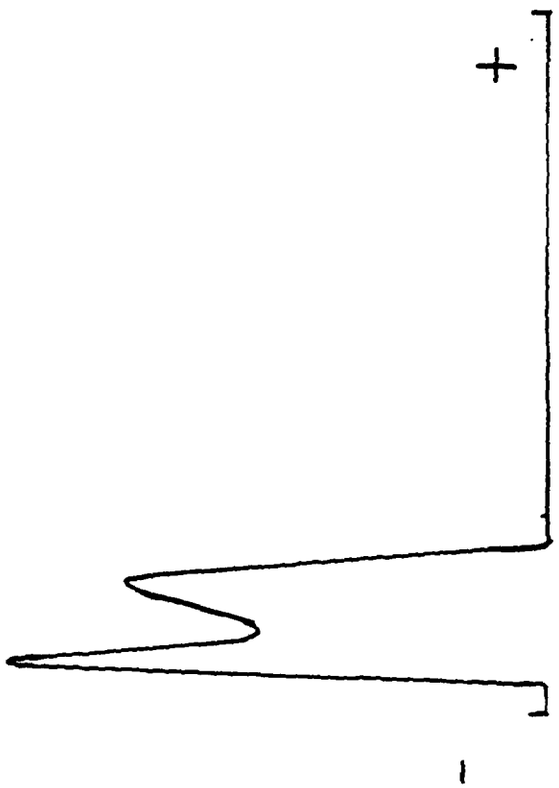
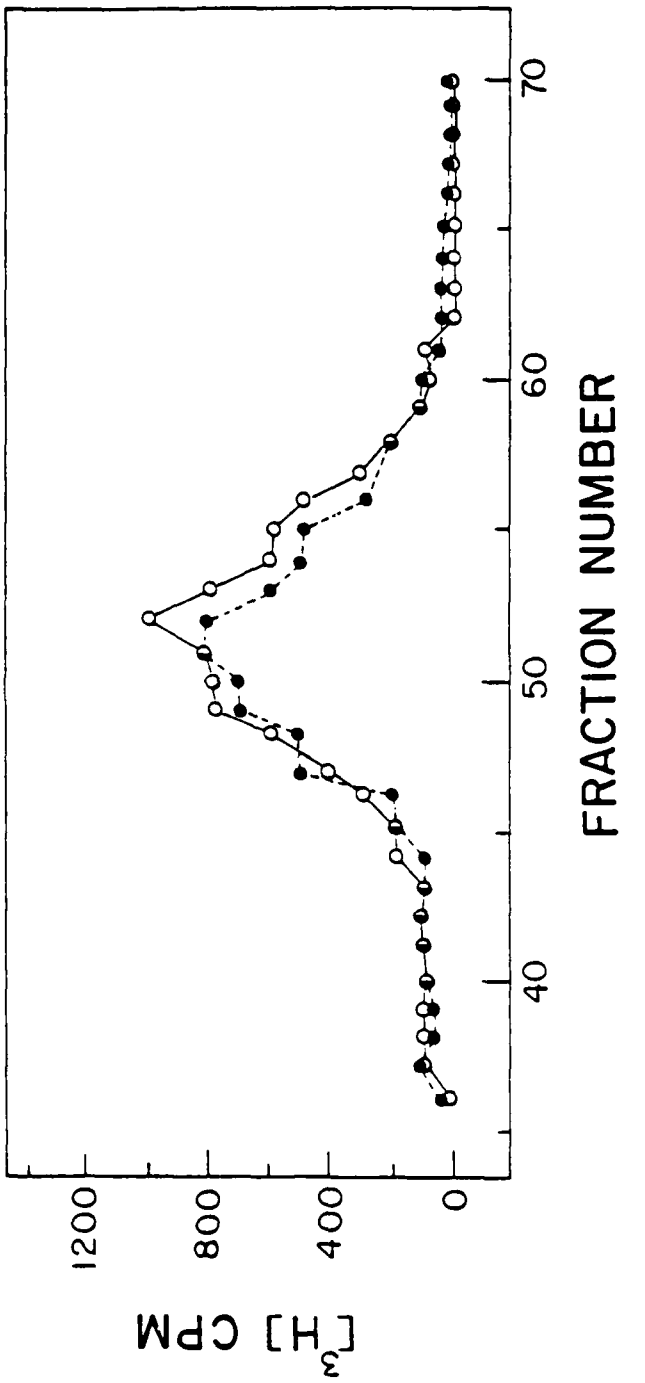


Fig. 22

Elution of RNA Polymerase on Sephacryl S-300

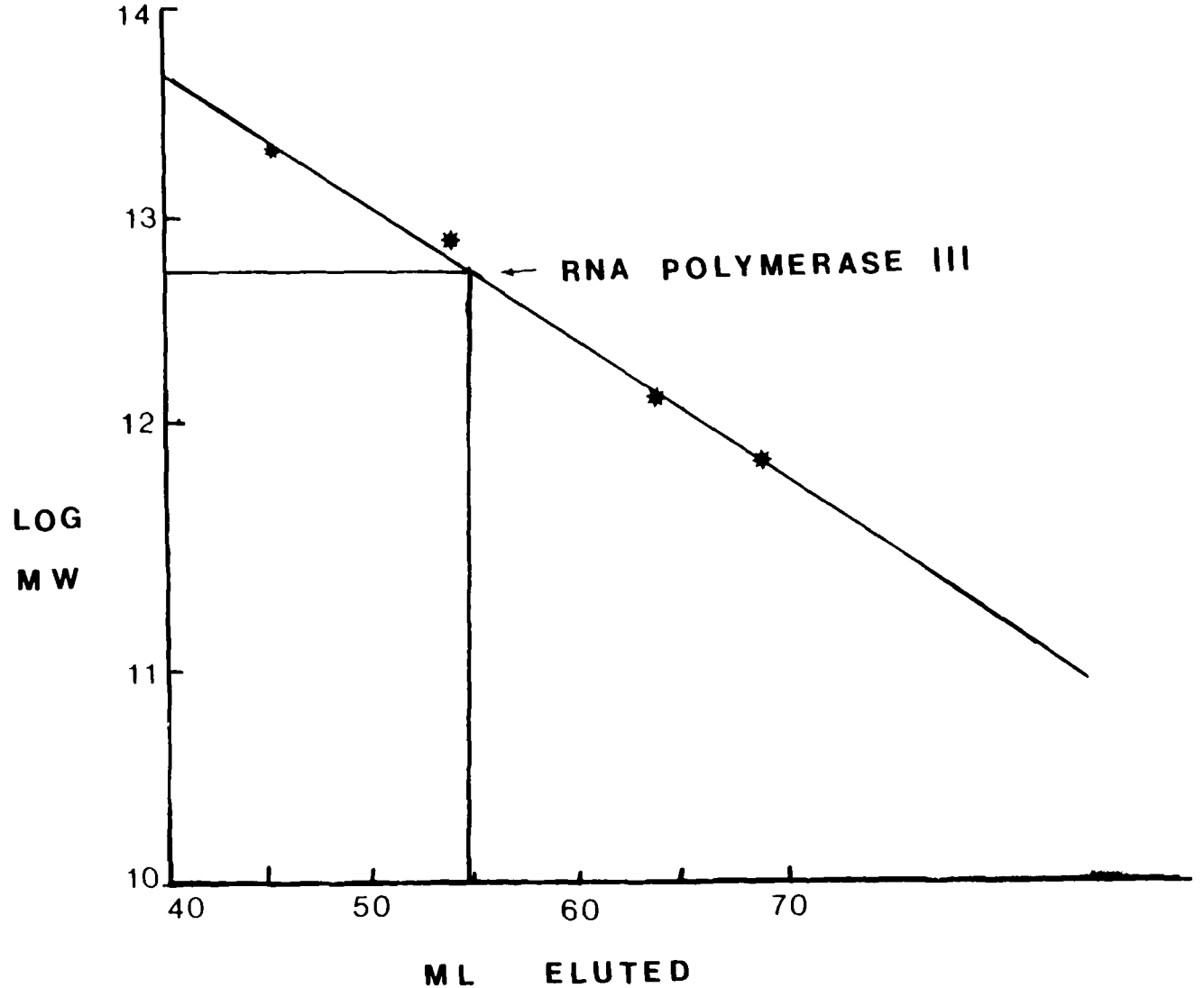


○ No α-Amanitin

● Activity in the presence of 1 ug/ml α-amanitin

ESTIMATION OF MOLECULAR WEIGHT OF RNA POLYMERASE

Fig. 23



Values for standard proteins ere plotted against their elution volumes. Standard mw of proteins were thyroglobin (669,000), apoferritin (443,000), B-amylase (200,000) and alcohol dehydrogenase (150,000). The elution position of RNA polymerase is indicated. (360,000)

Assay of RNA polymerase from the pooled fractions of a DEAE-Sephadex column revealed that the enzyme reaction was linear up to 20 min (Fig. 24). RNA polymerase isolated from DEAE-Sephadex chromatography showed 50% inhibition at a 4 μg/ml α-amanitin concentration (Fig. 25). The α-amanitin up to 20 μg/ml did not completely inhibit this enzyme. This same enzyme preparation dialyzed against Buffer A without metal ions was tested for optimum metal ion concentration in the RNA polymerase assay. Fig. 26 shows that the optimum concentration for $MnCl_2$ was 1 mM and that increasing the $MnCl_2$ concentration beyond 3 mM greatly inhibited the enzyme. The enzyme showed optimal activity with a $MgCl_2$ concentration between 2-4 mM, but this metal was only 14% as effective in enhancing optimal activity when compared to $MnCl_2$.

DEAE-Sephadex pooled fractions dialyzed against Buffer A without $(NH_4)_2SO_4$ were used to determine the optimum salt concentration for the RNA polymerase assay. As shown in Fig. 27, an $(NH_4)_2SO_4$ concentration between 0.02-0.06 M gave optimal RNA polymerase activity. Increasing concentrations beyond 0.1 M $(NH_4)_2SO_4$ inhibited the enzyme.

Since the purpose of our isolation of RNA polymerase was to provide an adequate system to test promising nucleotide analogs, we tested the effect of formycin A-triphosphate (FoA-TP) and cordycepin triphosphate on RNA polymerase isolated from DEAE-Sephadex chromatography. As shown in Fig. 28, FoA-TP appears to be a competitive substrate for the RNA polymerase. The addition of increasing concentrations of ATP (to 0.6 mM) in the presence of 0.4 mM FoA-TP relieves the competition, but does not completely reverse it. We have demonstrated previously that FoA-TP is incorporated into RNA and that the mRNA containing FoA nucleotides translates less efficiently than normal mRNA in L. mexicana promastigotes (17). We are in the process of determining the effect of tRNA containing FoA nucleotides on tRNA function.

Cordycepin triphosphate is known to affect RNA polymerase in a variety of organisms, but the exact kinetic nature of this inhibition is unknown (1). Cordycepin triphosphate inhibited the RNA polymerase in a linear manner from 10-60 μM, but increasing the concentration up to 100 μM did not completely inhibit this enzyme. This result may be due to more than one RNA polymerase species present in our preparation showing different sensitivities to this nucleotide, or the nucleotide may compete with the natural nucleotides (which are all present in the assay) and under these conditions was not able to bind to all the available sites.

The enzyme isolated has characteristics of true DNA-dependent RNA polymerase in that it requires DNA and all four nucleoside triphosphates for the synthesis of RNAase-sensitive products. The reported ammonium sulfate optima and metal ion optima, as well as relative activities of the enzyme with Mn^{2+} versus Mg^{2+} are similar to those reported for RNA polymerase III (9). Also, α-amanitin sensitivity and elution characteristics during chromatography indicate that this enzyme is RNA polymerase III (12).

RNA polymerase III is present in a variety of cells and tissues although its concentration is subject to great variability (9). Eukaryotic cells contain several distinct RNA polymerase proteins, which are localized in different subcellular fractions and play different functional roles in the cell. DNA-dependent RNA polymerase III represents one of the three major classes of nuclear RNA polymerases in eukaryotes. The class III RNA polymerases from a variety of cell types have been shown to have characteristic catalytic and chromatographic

Fig. 24

RNA POLYMERASE ACTIVITY IN RESPONSE TO TIME

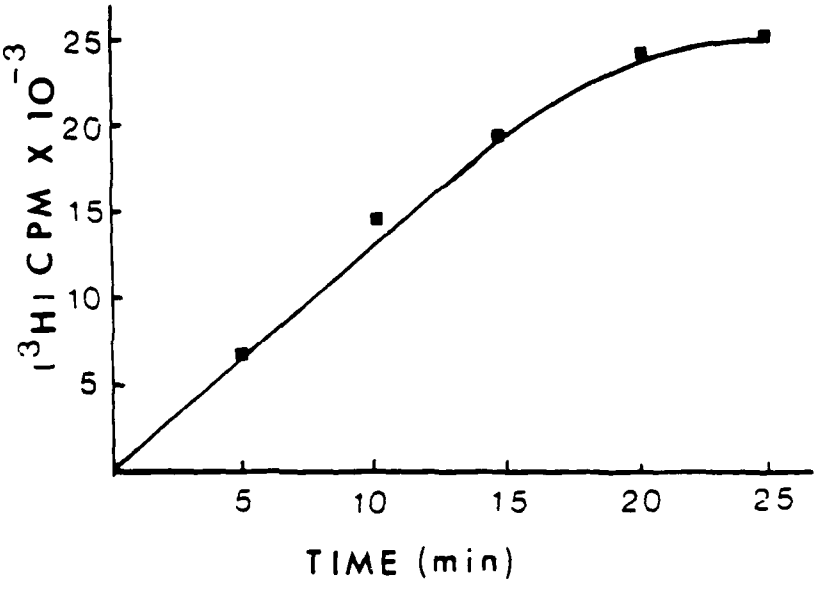


Fig. 25

Effect of α-Amanitin Upon the Activity of RNA Polymerase Eluted From DEAE-Sephadex Chromatography

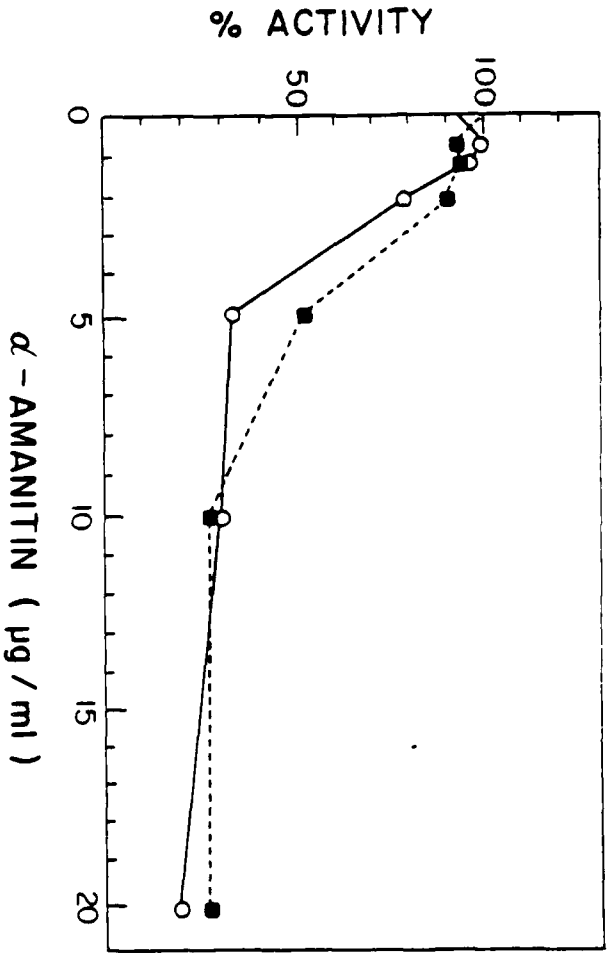


Fig. 26

Effect of Metals on RNA Polymerase Activity

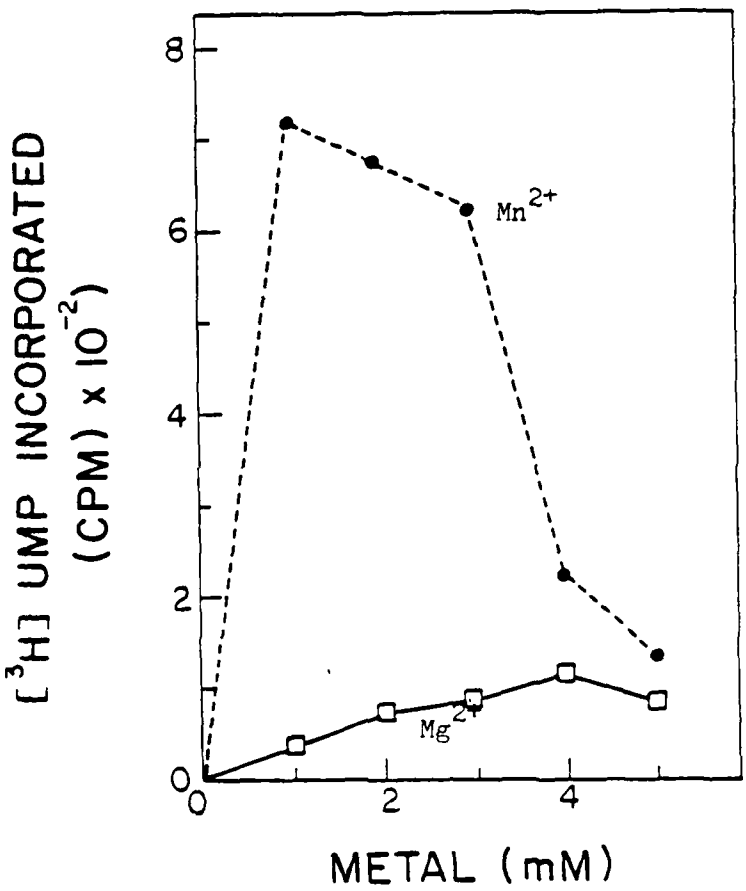


Fig. 22

Effect of Ammonium Sulfate Upon the Activity of RNA Polymerase Eluted From DEAE-Sephadex Chromatography

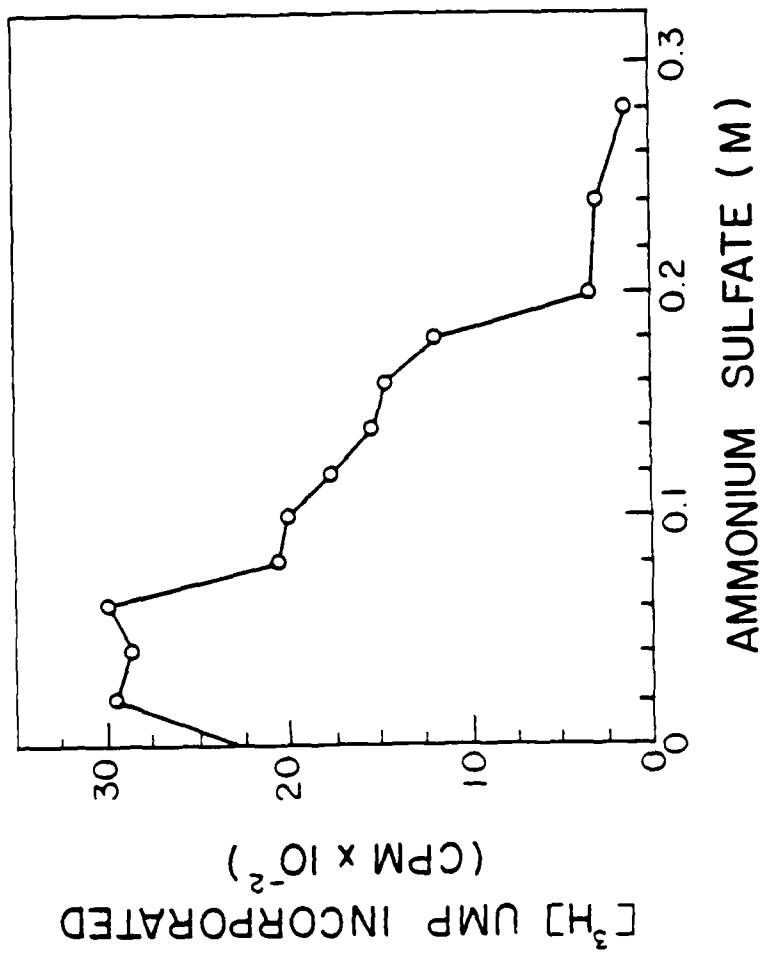
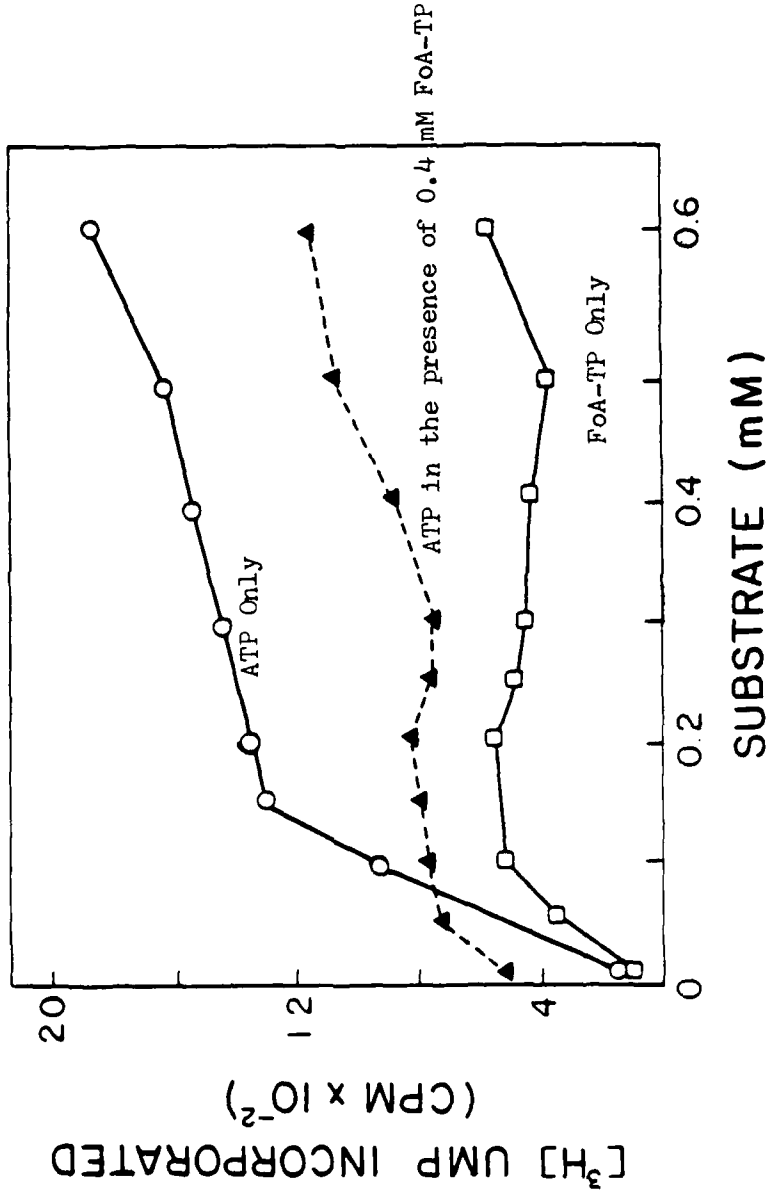


Fig. 2

RNA Polymerase Activity with Increasing ATP and FoA-TP Concentrations



properties and characteristic subunit structures, all of which distinguish these enzymes from the homologous class I and II RNA polymerases (9). The class III RNA polymerases have been implicated in the synthesis of tRNA, 5 sRNA, and other small cellular RNAs and in the synthesis of two small viral RNA species in adenovirus-infected cells (9).

We have tested the following compounds sent by WRAIR on isolated RNA and DNA polymerase. Our lack of inhibition probably results from the fact that many of these compounds are active when phosphorylated to the triphosphate.

Compounds Tested with No Effect on DNA Polymerase (500μm)

BK86124	Allopurinal riboside
BK74731	Oxoformycin
BK63005	3-B-D-Ribofurano sylpyrazolo-[4,3-d] pyrimidine-7-thione
BK48464	6-Aminoallopurinol Riboside
BK71338	Oxoformycin A

Compounds Inhibitory to DNA Polymerase at 500μm

BK63863	11.52%	Thiopurinol riboside

Compounds Inhibitory to RNA Polymerase at 500μm

BK74731	9.5%	Oxoformycin B
BK71338	13.7%	Oxoformycin A

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